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THE EFFECTS OF METHYLENE BLUE AND URETHANE (ETHYL CARBAMATE) UPON THE OXYGEN UPTAKE OF EMBRYONIC CELLS[1]

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That redox dyes, such as methylene blue, stimulate the oxygen uptake of most living cells seems well established (Barron, 1929; Barron and Hamburger, 1932). Cyanides, although depressants of respiration, are more or less completely antagonized by methylene blue in the case of most living cells (Barron, 1929; Barron and Hamburger, 1932; Brooks, 1932*a*, *b*, *c*, 1933; Green, 1940). Considerable variation in the quantitative relations between effective antagonistic concentrations of cyanide and methylene blue for different types of cells seems to exist (Bodine and Boell, 1936, 1937). Cyanides have been thought to inhibit respiratory processes by forming complex compounds with the iron- or copper-containing respiratory enzymes (Keilin and Hartree, 1938; Schultze, 1939), while the oxidative action of methylene blue is thought of as an oxidative dehydrogenation (Barron, 1929). Narcotics, such as urethane (ethyl carbamate), in low doses stimulate, while in higher concentrations they depress, the oxygen uptake of living cells (Bodine and Fitzgerald, 1948). Their chief site of action on the respiratory mechanism of the living cell is thought to be on the dehydrogenase parts of the respiratory system (Green, 1940). The effect of methylene blue upon the inhibitory action of urethane appears to be less clearly understood than for similar relations between it and cyanide (Barron, 1929). For certain types of cells, such as ova of sea urchin and starfish, urethane inhibits the action of methylene blue upon the oxidative processes (Barron, 1929).

Inasmuch as most of our data on the action of narcotics and methylene blue have been obtained from experiments on marine ova and other highly specialized cells, it seemed advisable to extend these observations to other forms. The present discussion, therefore, deals with results of experiments on the effects of methylene blue upon the action of urethane on the oxygen uptake of the grasshopper embryo, *Melanoplus differentialis*.

MATERIALS AND METHODS

Embryos of the grasshopper (*M. differentialis*) were dissected, free of yolk,

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from the eggs, and handled routinely as indicated in previous publications (Bodine and Boell, 1936, 1937). Solutions of methylene blue (National Aniline and Chemical Co., Inc.) and urethane (c.p. ethyl carbamate) were made up in phosphate-buffered Ringer solution (pH 6.8). Oxygen determinations were carried out with the usual Warburg techniques, using flasks of 5-ml. capacity at 25° C. One hundred embryos were used in each respiration vessel, and all concentrations recorded were those for final concentrations of reagents in the flasks and in contact with the embryos. Three sets of manometers (18 in all) were employed, and all data represent averages from such series, of which many have been run. Blank determinations with no embryos were carried out for all reagents employed.

Both active and blocked embryos were used, but, since results for each are qualitatively similar, no distinctions, except in a few cases, are pointed out in the text.

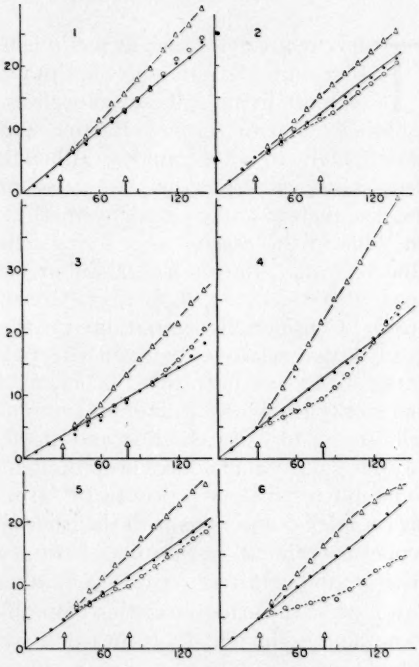
RESULTS

In studying the effects of urethane and methylene blue upon the oxygen uptake of embryos, it is essential to control not only final concentrations of each reagent in the respiration flasks but also the sequence of their additions to the flasks. The effects of methylene blue and urethane, administered separately, upon the respiration of grasshopper embryos have been previously noted (Bodine and Boell, 1936; Bodine and Fitzgerald, 1948, 1949). Inasmuch as results for all series of experiments are, in general, similar, a detailed discussion of individual experiments seems unnecessary at this time. A general description of the data obtained will therefore be given in connection with the figures. Results for all experiments herein reported are graphically shown in Plates I–IV.

a) METHYLENE BLUE FOLLOWED BY URETHANE

The addition of methylene blue to either active or blocked grasshopper embryos produces marked reversible increases in the oxygen uptake, as previously indicated (Bodine and Fitzgerald, 1949). Concentrations of 3×10^{-5} M and 25×10^{-5} have been employed in the present experiments, since marked reversible increases in oxygen uptake are obtained under such conditions (Bodine and Fitzgerald, 1949). The higher con-

PLATE I



Shows the effects of concentration and sequence of addition of methylene blue (M.B.) and urethane (ethyl carbamate, E.C.) on the oxygen uptake of embryos. *Abscissa*, time in minutes; *ordinate*, mm.^{3}O$_2$/100 embryos. Arrows indicate time of adding contents of side arms to respiration flask (no. of side arm refers to order in which their contents were added, unless otherwise stated).

FIG. 1.—Final concentrations = E.C. 0.05 M, M.B. 3×10^{-5} M.

KEY

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●.....	Ringer	Ringer	Ringer
○.....	Ringer	0.15 M E.C.	12×10^{-5} M M.B.+0.05 M E.C.
△.....	Ringer	9×10^{-5} M M.B.	0.20 M E.C.+$3.\times 10^{-5}$ M M.B.

FIG. 2.—Final concentrations = E.C. 0.10 M, M.B. 3×10^{-5} M.

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●.....	Ringer	Ringer	Ringer
○.....	Ringer	0.3 M E.C.	12×10^{-5} M M.B.+0.1 M E.C.
△.....	Ringer	9×10^{-5} M M.B.	.....

FIG. 3.—Final concentrations = E.C. 0.20 M, M.B. 3×10^{-5} M.

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●.....	Ringer	Ringer	Ringer
○.....	Ringer	0.6 N E.C.	12×10^{-5} M M.B.+0.2 M E.C.
△.....	Ringer	9×10^{-5} M M.B.	0.8 M E.C.+3×10^{-5} M M.B.

FIG. 4.—Final concentrations = E.C. 0.25 M, M.B. 25. $\times 10^{-5}$ M.

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●.....	Ringer	Ringer	Ringer
○.....	Ringer	0.75 M E.C.	100×10^{-5} M M.B.+0.25 M E.C.
△.....	Ringer	75×10^{-5} M M.B.	1 M E.C.+25×10^{-5} M M.B.

FIG. 5.—Final concentrations = E.C. 0.25 M, M.B. 3×10^{-5} M.

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●.....	Ringer	Ringer	Ringer
○.....	Ringer	0.75 M E.C.	12×10^{-5} M M.B.+0.25 M E.C.
△.....	Ringer	9×10^{-5} M M.B.	1 M E.C.+3×10^{-5} M M.B.

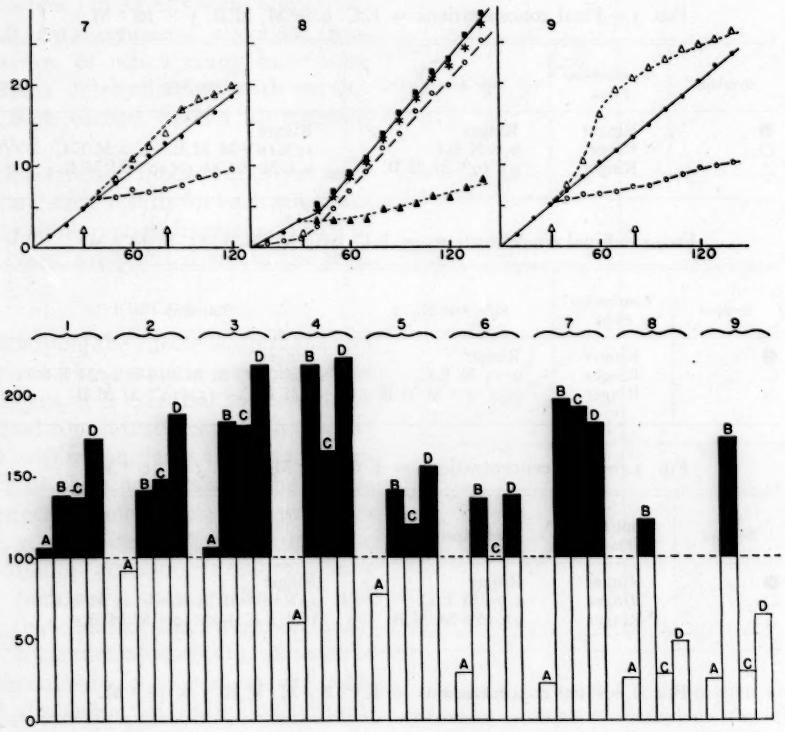
FIG. 6.—Final concentrations = E.C. 0.5 M, M.B. 3×10^{-5} M.

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●.....	Ringer	Ringer	Ringer
○.....	Ringer	1.5 M E.C.	12×10^{-5} M M.B.+0.5 M E.C.
△.....	Ringer	9×10^{-5} M M.B.	2 M E.C.+3×10^{-5} M M.B.

centration, 25×10^{-5} M, however, has been most extensively used, since it was desirable to have an excess of dye present in order to assure maximal stimulation effects with all combinations of reagents employed. Concentrations of urethane up to 1.0 M have been used, and these, in turn, except for the higher doses, are reversible ones (Bodine and Fitzgerald, 1948).

Urethane in concentrations up to 1.0 M, when added to embryos already maximally stimulated by the addition of methylene blue, has little, if any, effect upon the increased oxygen consumption of the organisms (Pls. I–IV). A more or less complete antagonism exists between the effects of the methylene blue and urethane when thus administered. In high concentrations of urethane (1 M), which represent the limits of reversibility of the narcotic effects, the antagonism is markedly reduced and finally disappears (Pl. I, figs. 7, 9, Pl. II, upper figs. 7, 9; lower figs. 8, 9; Pl. III, figs. 4, 5, 6). Within reversible and nontoxic limits,

PLATE II



Lower block figures. Shows effect of concentration and sequence of addition of methylene blue (M.B.) and urethane (E.C.) on the oxygen uptake of embryos (data represent average values). Oxygen uptake in Ringer taken as 100 per cent. *A* = urethane (E.C.); *B* = methylene blue (M.B.); *C* = E.C. followed by M.B.; *D* = M.B. followed by E.C., except for No. 7, where both were added together. Shaded portions represent values above controls (100 per cent); clear portions, below normal control rates.

Upper figures. Same as for Plate I.

FIG. 7—Final concentrations = E.C. 1 M, M B. 3×10^{-5} M.

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●	Ringer	Ringer	.....
○	Ringer	3 M E.C.	1 M E.C.+12×10^{-5} M E.C.
△	Ringer	9×10^{-5} M M.B	3×10^{-5} M M B.+2 M E.C.

FIG. 8.—Final concentrations = E.C. 0.5 M, M.B. 25×10^{-5} M.

Symbol	Respiration Flask	Side Arm
●	Ringer	.....
○	0.5 M E.C.	75×10^{-5} M M.B.+0.5 M E.C.
■	Ringer	75×10^{-5} M M.B.
+	Ringer	75×10^{-5} M M.B.+1.5 M E.C.*
▲	Ringer	1.5 M E.C.

* M.B. and E.C. were added together from side bulb.

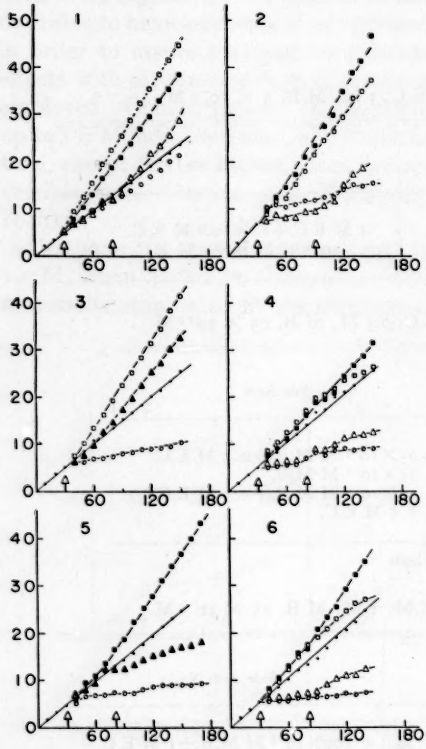
FIG. 9.—Final concentrations = E.M. 1 M, M.B. 25×10^{-5} M.

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●	Ringer	Ringer	.....
○	Ringer	3 M E.C.	100×10^{-5} M M.B.+1 M E.C.
△	Ringer	75×10^{-5} M M.B.	4 M E.C.+25×10^{-5} M M.B.

MOLAR CONCENTRATIONS

No.	E.C.	M.B.	E.C. and M.B.
1	0.05	3×10^{-5}	0.05+ 3×10^{-5}
2	0.10	3×10^{-5}	0.1 + 3×10^{-5}
3	0.20	3×10^{-5}	0.2 + 3×10^{-5}
4	0.25	25×10^{-5}	0.25+25×10^{-5}
5	0.25	3×10^{-5}	0.25+ 3×10^{-5}
6	0.50	3×10^{-5}	0.50+ 3×10^{-5}
7	0.50	25×10^{-5}	0.50+25×10^{-5}
8	1.0	3×10^{-5}	1.0 + 3×10^{-5}
9	1.0	25×10^{-5}	1.0 +25×10^{-5}

PLATE III



Shows the effect of concentration and sequences of addition of methylene blue (M.B.) and urethane (E.C.) on the oxygen uptake of embryos. *Abscissa*, time in minutes; *ordinate*, mm.^{3} O_2/100 embryos. Arrows indicate time of adding contents of side arms to respiration flask (no. of side arm refers to order in which their contents were added).

therefore, the stimulating effects of methylene blue on the oxygen uptake of embryos are not affected by the addition of urethane.

b) URETHANE FOLLOWED BY METHYLENE BLUE

As previously pointed out (Bodine and Fitzgerald, 1948), urethane in low concentrations produces stimulating effects upon the oxygen uptake of embryos, while in higher doses reversible inhibitory results are noted. Embryos, subjected to reversible inhibitory concentrations of urethane and then treated with stimulating doses of methylene blue, show methylene blue or antagonistic effects, but in a somewhat reduced fashion as compared to the effects of the initial application of methylene blue followed by urethane (see Pls. I–III). The inhibitory effects of urethane are still to a great degree antagonized by the methylene blue, but in a reduced manner. The sequence of the addition of either the dye or the narcotic to the embryo, therefore, seems to make some difference as far as the final results are concerned. Obviously, the physiological reaction brought about by the initial response to urethane conditions to a marked degree the quantitative response of the methylene blue subsequently added. As pointed out below, some parts of the respiratory mechanism are perhaps affected in a nonreversible fashion by the initial application of

FIG. 1.—Final concentrations = E.C. 0.1 M, M.B. 25×10^{-5} M.

KEY

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●	Ringer	Ringer	
△	Ringer	0.3 M E.C.	100×10^{-5} M M.B. + 0.1 M E.C.
○	Ringer	0.3 M E.C.	
□	Ringer	75×10^{-5} M M.B.	0.4 M E.C. + 25×10^{-5} M M.B.
■	Ringer	75×10^{-5} M M.B.	

FIG. 2.—Final concentrations = E.C. 0.5 M, M.B. 25×10^{-5} M.

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●	Ringer	.....	.....
△	Ringer	1.5 M E.C.	100×10^{-5} M M.B.+0.5 M E.C.
○	Ringer	1.5 M E.C.	0.5 M E.C.
□	Ringer	75×10^{-5} M M.B.	2 M E.C.+25×10^{-5} M M.B.
■	Ringer	75×10^{-5} M M.B.	25×10^{-5} M M.B.

FIG. 3.—Final concentrations = E.C. 0.5 M, M.B. 25×10^{-5} M.

Symbol	Respiration Flask	Side Arm
●	Ringer	Ringer
○	Ringer	1.5 M E.C.
□	Ringer	75×10^{-5} M M.B.
▲	Ringer	75×10^{-5} M M.B.+1.5 M E.C.

FIG. 4.—Final concentrations = E.C. 1 M, M.B. 5×10^{-5} M.

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●	Ringer	Ringer	.....
○	Ringer	3 M E.C.	.....
△	Ringer	3 M E.C.	20×10^{-5} M M.B.+1 M E.C.
□	Ringer	20×10^{-5} M M.B.	4 M E.C.+5×10^{-5} M M.B.
■	Ringer	20×10^{-5} M M.B.	.....

FIG. 5.—Final concentrations = E.C. 1 M, M.B. 25×10^{-5} M.

Symbol	Respiration Flask	Side Arm
●	Ringer	Ringer
○	Ringer	3 M E.C.
■	Ringer	75×10^{-5} M M.B.
▲	Ringer	75×10^{-5} M M.B.+3 M E.C.

FIG. 6.—Final concentrations = E.C. 1 M, M.B. 25×10^{-5} M.

Symbol	Respiration Flask	Side Arm No. 1	Side Arm No. 2
●	Ringer	Ringer	.....
○	Ringer	3 M E.C.	.....
△	Ringer	3 M E.C.	100×10^{-5} M N.B +1 M E.C.
□	Ringer	75×10^{-5} M M.B.	4 M E.C +25×10^{-5} M M.B.
■	Ringer	75×10^{-5} M M.B.	.....

the urethane, and this, in turn, probably renders them less affected by the subsequent addition of methylene blue.

c) METHYLENE BLUE PLUS URETHANE MIXED AND THEN ADDED TO EMBRYOS

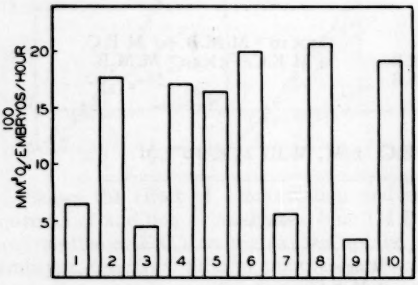
When antagonistic doses of methylene blue and urethane are mixed and then added together from the side arm to the respiration flasks, practically similar results are obtained as those for the methylene blue followed by urethane (Pl. I, fig. 8; Pl. II, lower fig. 7; Pl. III, figs. 3, 5).

d) *n*-BUTYL CARBAMATE AND METHYLENE BLUE

In addition to the extensive experiments on the effects of ethyl carbamate and methylene blue on embryos, others have been carried out with *n*-butyl carbamate and methylene blue. Results of these experiments, although not extensive, also show a marked inhibition of the effects of *n*-butyl carbamate by methylene blue (Pl. IV). An inspection of these figures shows that blocked or inactive embryos have a much greater stimulation by methylene blue than do the active ones. The inhibitory effects of the *n*-butyl carbamate are greater for the active than for the blocked organism. These results confirm earlier ones for cyanide and other compounds (Bodine and Boell, 1936, 1937) and extend the methylene blue antagonism to higher members of the homologous carbamate series.

In all the foregoing experiments, controls without embryos have been run in order to check possible reactions between the chemicals employed.

PLATE IV



Shows effect of normal butyl carbamate, methylene blue, and combinations of both on O_2 uptake of active and blocked embryos. *Ordinate*, mm.³ O_2/100 embryos/hour. *1*, diapause (blocked) embryos in Ringer (control); *2*, diapause (blocked) embryos $+25 \times 10^{-5}$ M M.B.; *3*, diapause (blocked) embryos +0.025 M *n*-B.C.; *4*, diapause (blocked) embryos +0.025 M *n*-B.C. $+25 \times 10^{-5}$ M M.B.; *5*, postdiapause (active) embryos in Ringer (control); *6*, postdiapause (active) embryos $+25 \times 10^{-5}$ M M.B.; *7*, postdiapause (active) embryos +0.025 M *n*-B.C.; *8*, postdiapause (active) embryos +0.025 M *n*-B.C. $+25 \times 10^{-5}$ M M.B.; *9*, postdiapause (active) embryos +0.04 M *n*-B.C.; *10*, postdiapause (active) embryos +0.04 M *n*-B.C. $+25 \times 10^{-5}$ M M.B.

DISCUSSION

The action of redox dyes and narcotics and especially of methylene blue and urethane upon the respiratory mechanisms of living cells has been much discussed, and out of such discussion have come varied theories as to their possible modes of action (Barron, 1929; Green, 1940). It seems impracticable to review such a vast amount of literature at this time, but certain results from the present experiments become of interest in the light of various ideas already noted for results from other types of biological materials in which methylene blue and urethane have been employed.

If surface forces operate in the normal respiratory mechanism of the living cell, adsorption and related phenomena should then be of paramount importance. Highly surface-active reagents, such as urethane, should therefore be significant in influencing surface reactions. As a matter of fact, methylene blue and urethane may well be assumed to be adsorbed by

living cells. For the grasshopper embryo, methylene blue is highly adsorbed, since the dye stains them an intense blue during the course of the experiments. The action of these two compounds on the oxygen uptake of sea-urchin and starfish eggs has been interpreted along such lines by Barron (1929). However, for marine ova urethane inhibits the stimulating action of methylene blue, and such a result is quite the opposite of that found for the grasshopper embryo. Embryos first treated with urethane and then with methylene blue do not show a complete antagonism between the two reagents. Such a condition may well be the result of an initial adsorption of the urethane, which cannot be completely displaced by the methylene blue. When the two compounds are added together, however, the methylene blue apparently is as readily adsorbed as when in pure solution and perhaps even at a faster rate than the urethane. A preferential adsorption may well be considered as taking place under such conditions. Other ideas, however, as to the fundamental action of inhibitors like urethane, as noted below, seem to make recourse to adsorption quite unnecessary in elucidating the basic antagonistic action of these compounds.

That narcotics act upon the dehydrogenase system of the cellular respiratory mechanism has been repeatedly pointed out (Barron, 1929; Green, 1940; Drabkin, 1948). The oxidative action of methylene blue on living cells belongs to the type of oxidative dehydrogenations, and data supporting this idea seem abundant (Barron, 1929). For the grasshopper embryo the antagonism between methylene blue and urethane depends upon the reversible nature of the cell, since an embryo irreversibly affected by urethane no longer reacts to added methylene blue.

According to the Warburg-Keilin theory of cellular respiration, methylene blue may act as an intermediate carrier, linking up the dehydrogenase with the oxidase parts of the system (Green, 1940). Urethane, on the same theory, supposedly attacks the dehydrogenase parts of the respiratory mechanism. Cyanides as well as azide are thought to involve the iron- and copper-containing oxidases (Keilin, 1933, 1936; Keilin and Hartree, 1938; Schultze, 1939). Results of studies on the respiratory quotients (R.Q.) of grasshopper embryos subjected to methylene blue show a rather constant value of 0.75, which is similar to that found for control, nontreated embryos (Table 1). Previous studies by Boell (1935) show quite clearly that, during the development of the grasshopper embryo, fats are used largely as sources of energy. Apparently no marked, if any, changes in the types of substrates utilized seem indicated when embryos are subjected to methylene blue. The normal respiratory mechanism seems in no way interfered with by the dye. For urethane, however, marked increases in the R.Q. values (0.094) are obtained (Table 2). Obviously, some changes in substrates utilized may be involved when urethane is added to the intact embryos.

TABLE 1*

EFFECT OF METHYLENE BLUE ON RESPIRATORY QUOTIENTS (R.Q.) OF EMBRYOS

Molar Conc. m.b.	Mm.³ O_2	Mm.³ CO_2	R.Q.
0.0	5.8	4.23	0.73
25×10^{-5}	11.8	8.85	0.75
20×10^{-5}	13.5	9.6	0.71
20×10^{-5}	12.5	9.75	0.78
20×10^{-5}	11.8	9.1	0.77
12.5×10^{-5}	11.5	8.85	0.77
12.5×10^{-5}	8.6	6.35	0.74
Av.			0.75

* All determinations of R.Q. values were carried out with the second method of Dickens and Simer, as described by Dixon (1943).

Values for the R.Q. obtained when urethane and methylene blue are mixed and then added to the embryos are strikingly similar to those for the methylene blue alone (Table 3). For other reagents, 2-4 dinitrophenol, pyocyanine, and azide, similar increases in R.Q. values for grasshopper embryos have been reported (Bodine and Boell, 1938; Carlson and Bodine, 1939). Ames, Ziegenhagen, and Elvehjem (1945), working with cell-free homogenates of mouse tissues, have also shown that the action of various inhibitors of respiration differ markedly, depending on which substrate or hydrogen donor is used as the initial substrate of the system. The values obtained for the R.Q. for the intact grasshopper embryo, therefore, seem clearly to indicate that marked changes in substrates or hydrogen donors may occur under the influence of urethane and other reagents as inhibitors of oxygen uptake.

TABLE 2

RESPIRATORY QUOTIENTS (R.Q.) OF EMBRYOS IN PRESENCE OF CARBAMATES

Stage	Molar Conc. of Carb.	Mm.³ O_2	Mm.³ CO_2	R.Q.
Blocked...	Control	5.80	4.2	0.73
Av.....	.....	.....	.....	0.75
	0.2 E.C.*	3.96	4.0	1.01
	0.3 E.C.	3.66	3.11	0.86
Av.....	.....	.....	.....	0.93
	0.3 E.C.	2.90	2.65	0.91
	0.03 *n*-B.C.†	2.26	1.92	0.85
	0.03 *n*-B.C.	2.56	2.64	1.03
Active....	Control	13.9	11.0	0.79
Av.....	.....	.....	.....	0.75‡
	.....	12.5	9.4	0.75
	0.3 E.C.	7.7	7.65	0.85
	0.3 E.C.	4.8	4.95	1.03
	0.03 *n*-B.C.	7.25	6.15	0.85
	0.03 *n*-B.C.	4.5	4.67	1.04
Av	.....	.....	.....	0.94

* E.C. = Ethyl carbamate.

† *n*-B.C. = Normal butyl carbamate.

‡ = Over-all average from many experiments. Data for blocked and active embryos indicated.

TABLE 3

RESPIRATORY QUOTIENTS (R.Q.) OF EMBRYOS IN PRESENCE OF 0.5 M ETHYL CARBAMATE + 25×10^{-5} M METHYLENE BLUE

Mm.³ O_2	Mm.³ CO_2	R.Q.
20.6	16.3	0.79
15.2	9.1	0.60
21.1	15.5	0.735
16.8	12.5	0.743
21.2	18.8	0.886
21.7	16.8	0.774
17.3	13.3	0.773
23.4	18.8	0.803
17.6	16.1	0.91
Av. 19.4	15.2	0.79

For hemolyzed red blood corpuscles of the horse, Runnström and Michaelis (1934) have shown that urethane added to methylene blue–treated systems, although cutting down on oxygen uptake, interfered but to a small and insignificant extent in the synthesis of phosphorus compounds. No marked antagonism between methylene blue and urethane was found for this system.

A rather simple explanation or theory as to the possible action of methylene blue and urethane upon the cellular respiratory mechanism of the grasshopper embryo can be advanced. If one accepts the idea of different substrates and hydrogen donors being available and used under the effects of different inhibitors and for the grasshopper embryo, data having already been given above showing the validity of such a concept, then one can reasonably expect an explanation through the action of these reagents upon the dehydrogenase and carrier systems of the cell. The accompanying diagram (Fig. 1), modified from Ames, Ziegenhagen, and Elvehjem (1945), illustrates this point of view for the grasshopper embryo. One may well

postulate the existence of various available substrate systems *A*, *B*, *C*, etc., and their accompanying dehydrogenase and carrier components, *X*, *Y*, *Z*, etc. Cytochrome *C* and cytochrome oxidase systems may be assumed to function in an orthodox fashion. In the dehydrogenase and carrier systems, there are normally, as in *A*, dehydrogenases and carriers actively making available, from substrate *S*, hydrogen for transport to the cytochrome system. Such a dehydrogenase and carrier system is susceptible to inhibitors, such as urethane. Methylene blue, when added to the normal cell, serves as additional carriers or hydrogen acceptors (*b*), supplementing those normally present (*a*) and utilizing the same substrate (*S*) as indicated by R.Q. results. Within reversible limits methylene blue is not affected by urethane in this capacity. A "respiratory ceiling" with methylene blue is reached when maximum utilization of substrate *S* through methylene blue and other normal carriers (*a* and *b*) takes place. Methylene blue, therefore, takes an active part in supplementing or adding to the carrier or hydrogen acceptor system already functioning in the normal cell. Methylene blue when acting as a carrier or hydrogen acceptor is not susceptible to the action of urethane as are the normal carriers (*a*) performing similar functions in respect to substrate *S*. Urethane, when added to the cell, changes the R.Q., indicating a utilization of a substrate (*S''*) differing from that (*S*) employed by the normal untreated cell. Different carriers or hydrogen acceptors (*a'*) for substrates (*S''*) utilized under the influence of urethane, are called into play, and these, in turn, are not affected by urethane within reversible concentrations. Methylene blue, therefore, when added to the normal embryonic cell, supplements or adds to the already active system of hydrogen acceptors, causing a maximum utilization of substrate and a subsequent increase in oxygen uptake by the system. Urethane added to the methylene blue–treated cells inhibits the normal hydrogen acceptors but does not affect the methylene blue system, and hence the inhibitory action of urethane is antagonized. Urethane added to the normal cell inhibits all normal hydrogen acceptors and then employs another nonnarcotic-sensitive set to make available another substrate. Reduced oxygen uptake is then conditioned by the number, availability, and capacity of the new system, substrate + hydrogen acceptors, as indicated in Figure 1.

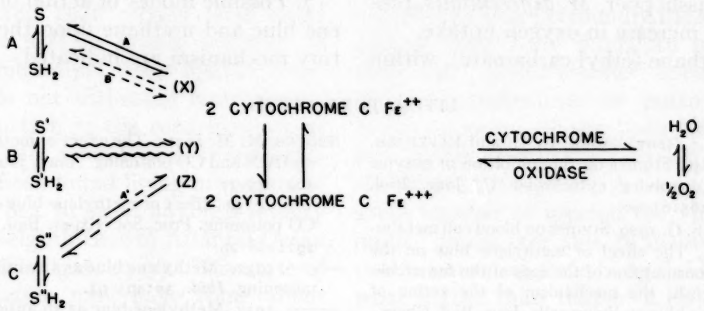


FIG. 1.—A schema, showing possible action of methylene blue (M.B.) and urethane (E.C.) on the respiratory mechanism. For further explanations see text.

Existing data for the grasshopper embryo seem to indicate that the increased oxygen uptake due to methylene blue is both CN- and CO-sensitive (Bodine and Boell, 1936, 1937). For the ova of sea urchins and starfish, quite the reverse has been reported (Barron, 1929; Brooks, 1932*b*). It seems reasonable, therefore, to assume, in view of existing data, that differences in the basic respiratory mechanisms may be involved in enzyme inhibition.

SUMMARY

1. Methylene blue added to embryos of the grasshopper, *M. differentialis*, produces an increase in oxygen uptake.

2. Urethane (ethyl carbamate), within reversible limits, does not inhibit the action of methylene blue.

3. The sequence of additions of methylene blue and urethane quantitatively affects the oxygen uptake of the embryos.

4. The addition of methylene blue has no effect on the normal respiratory quotient (0.75).

5. Urethane raises the respiratory quotient from 0.75 to 0.94.

6. Urethane and methylene blue mixed and then added to the embryo give respiratory quotients characteristic of methylene blue alone.

7. Possible modes of action of methylene blue and urethane upon the respiratory mechanism are indicated.

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METABOLIC REACTIONS OF CLAMS' TISSUES TO CHANGE IN SALINITY

I. CILIARY ACTIVITY, NARCOTIC AND CYANIDE EFFECTS, AND RESPIRATORY QUOTIENT

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IT IS well known that some marine animals can become adapted to life in a brackish or nearly fresh-water environment and that certain fresh-water species are able to invade brackish or strong saline waters. Most marine invertebrates do not withstand more than a slight reduction in the concentration of sea water. Yet some typical marine species have been found living in reaches of the Baltic Sea, in water having a salinity about one-eighth that of Atlantic Ocean water (Välikangas, 1933). This state of acclimatization may have come about through gradual adjustment to lower concentrations, during the colonization of inland waters, or by the appearance through mutation of races more resistant to low salinity. The possibility of such adaptation occurring in individuals was demonstrated as early as 1816 by Beudant (see Schlieper, 1933). He was able to induce various marine mollusks to live in fresh water, after transferring them successively to more dilute sea water, in experiments lasting over 5 months; and he brought about the adaptation of fresh-water mollusks to life in sea water in about the same time. More recently, investigators have obtained similar results in other species.

In order to explain the mechanism involved in adjustment to rapid changes in salinity, attention has been directed to the study of brackish-water forms of animal life. Estuarine animals are exposed to large changes in the saline concentration of the environment, which might be expected to alter profoundly the salt content of their body fluids. In many of these the "poikilosmotic" species, the saline concentration—or potential osmotic pressure—of the body fluids follows rather closely the concentration of the salts in the medium. A comparatively small number of species, the "homoiosmotic" forms, are able to maintain the concentration of their body fluids somewhat higher than that of a diluted sea water. Actually, these do not all possess equal powers of regulation. Numerous investigations dealing with the adaptation of animals to fresh and saline waters have been undertaken to explain the mechanism of osmotic regulation and the function of special organs in the process. These have been reviewed by Schlieper (1930, 1935), Pantin (1931), Krogh (1939), and Beadle (1943).

Since the maintenanceof hypertonicity in the body fluids, or cells, by an animal in a hypotonic medium would involve energy expenditure, one would expect it to be accompanied by an elevated respiratory metabolism. In many instances the oxygen requirements of fresh-water animals are known to be higher than those of marine representatives of the same, or closely related, species. Moreover, several marine and brackish-water

species studied have been found to have an increased oxygen consumption when transferred to a more hypotonic environment. The higher rate in some of these forms was sustained, while in some it was only temporary. Certain species, however—including at least one (*Eriocheir sinensis*) which is homoiosmotic—showed no change in respiratory rate in a diluted medium; others showed a definite decrease. It is probable that some in this last group were already living at suboptimal concentrations of sea water. We do not yet know how many species of aquatic animals undergo an increase in respiration when placed in a diluted medium.

Our knowledge of the factors involved in the stimulation of respiration by hypotonic solutions is also incomplete. No explanation has been generally accepted as accounting for the conditions in all species studied, as may be illustrated by some of the earlier investigations. Tarussov (1927) found, according to Schlieper (1929), that in *Nereis diversicolor* the respiration was augmented during the first few hours in a more hypotonic medium and was depressed for a few hours in a hypertonic medium, gradually returning in each case to its original rate. He attributed these effects to hydration and dehydration of the tissues. Schlieper (1929) reported that in *Carcinus maenas* there was an increase in respiration in a diluted medium, without any initial hydration of blood or tissues, whereas in *N. diversicolor*, there was increased respiration and hydration (swelling) amounting to 34 per cent, which persisted after the respiratory rate began to fall. He attributed the rise in rate in both species to the osmotic work performed in opposing the entrance of water, the fall in rate (*Nereis*) to a harmful effect of the hypotonic sea water. That a hydration of tissues (cell colloids) may play an important part in the stimulation of respiration by hypotonic sea water was later recognized as probable by Schlieper (1931), since he found that the oxygen consumption of the excised gills of *Mytilus edulis* was augmented by isotonic solutions of single salts (NaCl, KCl, or $CaCl_2$) and that of *Gammarus locusta* by isotonic NaCl. His earlier observation (1929), that pieces of *Mytilus* gill showed a rise in respiratory rate in diluted sea water, also pointed to factors other than muscular and renal activity. Beadle (1931) ruled out the influence of muscular activity upon respiration in two species of *Nereis* and in *Gunda* (*Procerodes*) *ulvae*, by using a narcotic—chloretone. He demonstrated that swelling occurred in sea water and that there was an increased rate of respiration, which was temporary in *Nereis* but apparently permanent in *Gunda*. The small increase in respiration in *N. cultrifera*, compared with that in *N. diversicolor*, was correlated with its feeble power of osmotic regulation. He found, too, that anaerobiosis and exposure to KCN—conditions which retard respiration—resulted in greater swelling, thus indicating a loss of ability to maintain hypertonicity in the body fluids. Schwabe (1933) also found that *C. maenas*, in brackish water at low oxygen tensions, lost much of its power of osmoregulation: there was a fall in blood osmotic pressure which was reversible, when the animals were returned to aerated sea water. The temporary increases in oxygen consumption in *Nereis* mentioned above (Schlieper, Beadle) are considered by Krogh (1939) to be within the limits of experimental error and not significant. He notes the absence of a higher respiration after a period long enough for acclimatization to the lower salinities, when osmotic work would be done.

There are certain, more direct, lines of evidence which indicate that energy is expended is osmotic regulatory processes.

The excretion of hypotonic urine by the kidney of vertebrates (and some invertebrates) living in fresh water, or after drinking it in large amounts, suggests that osmotic work is involved in reabsorption of salts in the kidney. Marine teleost fishes maintain the osmotic pressure of the blood at a considerably lower level than that of the environment, although they ingest sea water. It has been found that sea water is absorbed from the intestine into the blood and that regulation is accomplished by the secretion of salts through the gills to the exterior (Smith, 1930, 1932; Keys, 1931, 1933). Bateman and Keys (1933) found that the isolated gills of the eel, filled with Ringer's solution, consumed oxygen at a 43 per cent higher rate and, filled with blood, at an 80 per cent higher rate when immesed in five-sixths sea water as compared with one-ninth sea water. This seems to show that extra energy is required for the secretion of salt against an osmotic gradient. The "chloride cells" discovered by Keys and Willmer (1932) in the eel's gill may function in this connection.

The investigations of Sylvia Pieh (1936), like those of Schlieper, were interpreted as supporting the hydration theory as it applies to tissues. She found that the gills of *M. edulis* and of *C. maenas* undergo swelling in hypotonic sea water or in an isotonic NaCl solution and that in each of these solutions an increase in respiration occurs (*Carcinus*). When the gill tissue of *Carcinus* was exposed to cyanide in either solution, the respiration was inhibited but not the swelling. In another species of crab, *E. sinensis*, which has the same rate of respiration in fresh water as in sea water (Schwabe, 1933), she found that the gills from fresh-water animals did not contain any more water than did those from marine animals. It had previously been shown, in *Mytilus* (Bauxin, 1931) and in *Carcinus*, that there was a higher rate of respiration in animals transferred to dilute sea water than in the sea water in which they had lived.

In earlier experiments (Hopkins, 1946) it was found that the rate of oxygen consumption of the gill of *Venus mercenaria* was about one-third greater in sea water of density 1.015 than in natural sea water (1.025); and this was true whether the animals had been living in sea water at the lower or at the higher concentration. The increased respiratory rate was therefore not a temporary effect of the dilution, as was the case for *Mytilus* gill (Schlieper, 1929). The clam's gill can be removed with slight injury and survives well in sea water; moreover, the lamellae are porous and ciliated, allowing ready access of dissolved oxygen to all the tissue. Hence it is a favorable object for investigating the effects of environmental changes on respiration.

It seemed important at the outset to ascertain what part, if any, ciliary activity might play in the increased respiration of gill in dilute sea water. The role of other factors, such as osmoregulation or hydration, could then be more easily evaluated. In order to determine whether other tissues respond like the gill to dilution of the sea water, the oxygen consumption of mantle and muscle tissues was also measured. Determinations of the respiratory quotient were made originally to find what relation it might have to the seasonal variations in respiration described earlier (1946) and to observe any effects of mechanical stimulation due to shaking the respirometers. Although no seasonal differences in R.Q. were found, the results are included in this paper because of the paucity of such data for invertebrates and for any light they may throw upon the nature of the hypotonic stimulation of respiration. An R.Q.

approaching 1.0 under these conditions might indicate increased oxidation of tissue carbohydrate but would not necessarily mean that osmotic work was performed.

METHODS

Ciliary activity was estimated by observing the movement of pieces of gill lamella, just covered with sea water, in a flat culture dish. Vertical strips were cut, approximately 3 × 10 mm. in size, leaving the ventral ends intact. The propulsive action of the frontal cilia toward the ventral border caused the gill strip to move in the opposite direction. At least four determinations were made upon each, using a lens and millimeter scale. Usually several pieces were placed in one solution, and the average rate per minute was estimated. For comparison with the effects of dilute sea water, chloretone—a narcotic which depresses ciliary activity—was also used.

The measurements of oxygen consumption were made with differential volumeters (Hopkins, 1946). As a rule, the experiments upon the effects of sea water and reagents, were matched—i.e., "aliquot" portions of a tissue were taken from one clam. From 5 to 8 pieces of gill were used; about 10 pieces of mantle tissue, from the thin central part of the mantle; and about the same number of muscle sections, from the red parts of the adductors. The weights of these tissues, dried at 105° C., were roughly 25, 20, and 100 mg., respectively. The measurements of O_2-consumption were made in 1.5–2.0 ml. of solution, at 20°, and with oxygen when not otherwise stated. A shaking rate of about 60 cycles per minute was used.

The respiratory quotient was determined by the three-chamber method of Warburg (Dixon, 1943). Pieces of gill, 2–4 mm. wide, were cut from the lamellae of one clam (and occasionally two) and were then placed successively in three dishes, in sea water adjusted to pH 6.7. The pieces (about seven in each portion) were washed twice in this medium and transferred to three respirometer flasks, each containing 0.5–0.7 ml. of the same. Oxygen consumption was measured in the usual way in one instrument, with 5 per cent KOH to absorb the CO_2. The pre-formed CO_2 was determined in a second instrument, after 15 minutes from the start of the experiment, and the pre-formed *plus* formed CO_2 in a third instrument, about 4 hours later, so as to coincide with the final reading of O_2-consumption. The CO_2 was liberated from the tissue and sea water by spilling 0.2 ml. of 2 M HCl from a side-bulb into each vessel. Corrections were made for the CO_2 dissolved in the fluid in a vessel, after each determination of formed CO_2, according to the relative volume of gas and liquid phase (Fenn, 1927). This correction raised the average figure for CO_2, and hence the R.Q., about 4 per cent. Oxygen was used in nearly all experiments, but no significant difference in R.Q. was found in the few in which air was used.

RESULTS

Ciliary activity.—Sea water diluted to 60 per cent, contrary to expectation, did not increase the activity of the frontal cilia (Table 1), although it augments gill respiration. In all except two cases it retarded their activity, especially during the first hour. The depressant effect lasted many hours, exceeding the time during which oxygen determinations were made (Tables 2–4). There seemed to be some recovery from inhibition in the diluted medium in the last two experiments. Wells and his collaborators (1940), working with the gill of *M. edulis*, reported inhibitory effects of changes in

salinity upon the cilia, which were followed by accommodation.

The action of chloretone upon ciliary activity, like that of dilute sea water, was inhibitory. The rate was reduced roughly by one-fourth in a 0.05 per cent solution, and by two-thirds in a 0.10 per cent solution, in sea water. It is significant in this connection that the O_2-consumption of the gill was found to be 11 and 7 per cent higher in sea water containing chloretone at these respective concentrations than in sea water (Table 2). Another point of interest is that the increase in oxygen respiration, which occurred in the clam's gill in sea water of density 1.015, was hardly affected by the presence of 0.05 per cent chloretone. The rate was 39 per cent higher than that in natural sea water (Table 2, first series), as compared with a 41 per cent increase in dilute sea water without chloretone (third series).

TABLE 1

CILIARY ACTIVITY OF GILL LAMELLAE FROM THE CLAM, *Venus mercenaria*, IN SEA WATER AT DIFFERENT SALINITIES AND TREATED WITH CHLORETONE

Sea-Water Density; Chloretone Conc., in S.W. (1.025)	No. of Gill Strips	Rate of Movement (Mm/Min) at Different Intervals of Time after Treatment (Hours)				
		0.0	0.5	1.0	2.0	3 Hours or Later
S.W., 1.025	1	24.4		27.0		
S.W., 1.015	1	11.5		19.7		
S.W., 1.025	3	7.4				
S.W., 1.015	6		10.3			
Chl., 0.05%	4	6.9				
S.W., 1.025	7	11.4			13.6	
S.W., 1.015	6	4.1			8.3	
S.W., 1.025	3	12.0				
S.W., 1.015	3	12.8				8.3 after 6 hours
S.W., 1.025	5*	16.7				
Chl., 0.05%	4	7.5			5.5	
S.W., 1.025	4	12.0		13.6		
S.W., 1.015	6	5.4			9.1	
Chl., 0.10%	4		6.3		8.8	
S.W., 1.025	6	10.0				10.0 after 5 hours
S.W., 1.015	7		9.4			6.9 after 5 hours
Chl., 0.10%	5	2.2				2.3 after 5 hours
S.W., 1.025	6	8.6				
S.W., 1.015	6	2.2				
Chl., 0.10%	6	3.3				
S.W., 1.025	7	16.2				15.8 after 10 hours
S.W., 1.015	7	4.0				10.0 after 10 hours
Chl., 0.10%	5†	2.4				
S.W., 1.025	7	9.5				15.4 after 3 hours
S.W., 1.015	7	3.0				12.8 after 4 hours

* Transferred, after 6 hours in sea water 1.015, to sea water 1.025.
† No movement observed in two other pieces of gill.

The effects of dilution upon the respiration of other tissues.—In sea water of density 1.015 there was a 12 per cent rise in respiratory rate for the mantle and a

15 per cent fall in rate for muscle (Table 2). It is quite possible that the thin mantle membrane, which becomes wrinkled after excision, is thereby rendered more permeable to water. The same might be said of the muscle; for, although it was sectioned parallel to the fibers, some cell damage could hardly be avoided. Since the gill and mantle are epithelial tissues, they are, perhaps, functionally differentiated to resist the penetration of water into the body (or cells); and this may account for their positive metabolic response in a hypotonic medium, unlike that of muscle. Schlieper is reported to have found, however, that excised crab muscle showed a large increase in O_2-consumption in a hypotonic medium (Pieh, 1936, footnote).

The influence of cyanide.—The effect of millimolar cyanide upon the respiration of the clam's tissues was quite pronounced in the case of mantle and muscle, being about 60 per cent in each of these. The gill responded differently according to season. Some inhibition of respiration occurred during the cooler months. as shown by the experiments in the second series in table 2 and, to a greater extent, in some tests made in late

TABLE 2*

OXYGEN CONSUMPTION OF THE GILL OF THE CLAM, *Venus mercenaria*, AND EFFECT OF CHLORETONE IN NATURAL AND DILUTE SEA WATER; ALSO, RESPIRATION OF VARIOUS TISSUES TREATED WITH CYANIDE

Sea Water Density 1.025	Chloretone 0.05%, in S.W., 1.025	Chloretone 0.05%, in S.W., 1.015	Chloretone 0.10%, in S.W., 1.025	Date
(3) 1,056	(3) 1,179	(3) 1,466	(2) 1,127	
Sea Water 1.025	Sea Water 1.015	HCN (10^{-3} M) S.W., 1.025	HCN (10^{-3} M) S.W., 1.015	
	Gill			
(3) 1,200		(3) 1,176		Aug. 15–Sept. 4†
(5) 1,287	(5) 1,819	(5) 933	(5) 1,225	Sept. 15–20†
(8) 1,176‡		(8) 1,186‡		July 22–Sept. 6
(5) 1,237		(5) 1,282	(5) 1,608	July 22–Sept. 6
	Mantle			
(5) 773‡	(4) 842‡	(5) 308‡		July 26–Sept. 2
(3) 768	(3) 860	(3) 298	(3) 274	July 27–Aug. 12
	Muscle			
(3) 71.2	(3) 60.3	(3) 27.8		July 29–Aug. 3

* The volumes of oxygen (cubic millimeters per hour and gram dry tissue) are reduced to 0° C, dry. Each figure represents the average rate for the number of measurements given in parentheses. Experiments on the same line are matched where an equal number is indicated.

† NaCN added to tissue after previous determination of O_2-consumption in sea water; KOH in side-bulb. The other measurements of cyanide effects were made according to the calcium-cyanide method of Robbie (1946).

‡ Includes the experiments listed in the line below.

autumn and winter, not recorded here. The slight inhibition indicated in the first cyanide experiments on gill (Table 2) may be the result of starting the measurements of O_2-consumption in cyanide ½ hour after the control readings, upon the same tissue in sea water, had been completed, whereas, with the Ca-cyanide method, simultaneous readings were made on different samples of tissue. It will be noted that, in the September series, a 41 per cent increase in O_2-consumption in sea water of density 1.015 was subject to complete inhibition by cyanide (fourth column of table). The inhibition in dilute sea water during the summer (last series) was quite small. Cyanide inhibited the respiration of the mantle in dilute sea water from a potential high level of 860 to a rate even lower than that in natural sea water with cyanide—or by 68 per cent. Solandt, Solandt, and Gerard (1934), also working with the gill of *V. mercenaria*, reported a 50 per cent inhibition of O_2-consumption in M/550 cyanide and of 85 per cent in M/110 cyanide. Chapeau (1932) found that in the Portuguese oyster, *Gryphea angulata*, M/1,000 KCN inhibited the respiration of mantle, gill, and muscle about 50 per cent, and that of hepatopancreas nearly 80 per cent.

Salinity of the sea water.—An attempt was made to determine whether there was an optimal concentration of sea water, which, from the standpoint of economy of energy, should be the one at which the O_2-consumption of the tissue is lowest. As shown in Table 3, the rate for the gill was inversely proportional to the density of the sea water between 1.030 and 1.012+, for clams adapted to sea water 1.024. At density 1.010 the O_2-consumption was almost equal to that at 1.012+, but the results were discarded because some disintegration of tissue occurred.

TABLE 3

OXYGEN CONSUMPTION OF CLAM'S GILL (CU MM/GM DRY WT/HR) IN DIFFERENT CONCENTRATIONS OF SEA WATER, IN ISOTONIC NaCl SOLUTION, WITH AND WITHOUT SEA WATER AND AFTER THE ADDITION OF CALCIUM

Sea Water 1.030	Sea Water 1.025	Sea Water 1.012+	NaCl, 3.1 Per Cent (1.023+)	NaCl, 3.1 Per Cent +$CaCl_2$, 0.1 Per Cent (1.023+)	NaCl, 50 Sea Water, 50 (1.024)	NaCl, 60 Sea Water, 40 (1.024)
	(7) 1,128*	(7) 1,631*				
	(4) 1,052	(4) 1,613	(4) 1,545	(4) 1,100		
(5) 999†	(5) 1,120†	(3) 1,656†			(3) 1,077†	(2) 1,031†
(4) 999	(4) 1,078					

* Includes the four determinations listed on the line below.
† Experiments not closely matched with the others on same line.

Effects of salt solutions.—When the clam's gill was placed in a solution of pure NaCl, nearly isotonic with sea water 1.025, there was a 47 per cent greater rate of O_2-consumption than in sea water (Table 3). This stimulating effect was almost completely offset by the presence of $CaCl_2$ in 0.1 per cent concentration (equivalent to that in sea water). The addition of sea water to an isotonic NaCl solution, up to 40 per cent of the total volume, was sufficient to reduce the gill respiration to a rate below that found in sea water. In this case the antagonism was evidently not due to calcium alone, since it was present in a concentration less than half that in sea water.

The respiratory quotient.—The results of measurements of the R.Q. made at two widely different shaking rates and in two concentrations of sea water are summarized in Table 4. Both rate of respiration and R.Q. were apparently greater at the higher rate of shaking. The differences in the R.Q. values for the two shaking rates, however, are not statistically significant. Whatever effect the rapid shaking may have had, it was probably exerted by stimulating the tissue mechanically rather than by promoting oxygen diffusion, since the respirometers were swung in a wide arc and the volume of medium was small compared with the amount of tissue. Moreover, the O_2-consumption of gill is not much greater with oxygen than with air (Hopkins, 1946).

The effects of a reduced concentration of sea water were more obvious. The respiratory rate increased up to 57 per cent in the first two series; in the larger series (3–6) it increased 41 per cent during the spring and 49 per cent during late May and June. The R.Q. increased 8–9 per cent in sea water 1.015 over that in natural sea water. Comparing the values obtained at corresponding seasons, one observes significant differences. For the

TABLE 4*

EFFECTS OF REDUCED CONCENTRATION OF SEA WATER, AND OF THE RATE OF SHAKING, UPON OXYGEN CONSUMPTION AND RESPIRATORY QUOTIENT, AT 20°, IN THE GILL OF *Venus mercenaria*

Density of Sea-Water Medium	No. of Experimental Series and Time of Year	No. of Determinations, O_2-Consumption and R.Q.	Slow Shaking		Fast Shaking	
			Oxygen Consumption	R.Q. and Probable Error	Oxygen Consumption	R.Q. and Probable Error
			31 per Min.		93 per Min.	
1.025	(1) June	3 pairs	1,528	0.865	1,618	0.909
1.015	(2) May–June	3 pairs	2,403	0.930	2,725	0.960
	(3) Mar.–Apr.	10 pairs	1,418	0.866 ±0.012	1,482	0.889 ±0.012
1.025			31–60 per Min.			
	(4) June	6†	1,561	0.867 ±0.013		
			45 per Min.			
	(5) Mar.–Apr.	16	1,998	0.946 ±0.011		
1.015			30–60 per Min.			
	(6) May–June	7†	2,332	0.936 ±0.012		

* Oxygen volumes (cu mm/gm dry wt/hr) are reduced to 0° C., dry. Tissue from same animals was used in each determination of the R.Q., and in each pair.

† Includes three determinations listed above with the paired experiments.

spring series (3, 5) the difference is 0.080 ± 0.016; and, for the summer series (4, 6), it is 0.069 ± 0.018. The difference in the first case is five times its probable error, and in the second case nearly four times.

It is interesting to compare these results with those obtained by other investigators using Lamellibranch gill. Gray (1924), working with the gill of *M. edulis*, found an average R.Q. of 0.84. For the gill of *Dreissensia*, a fresh-water mussel, Wernstedt (1944) reported a mean value of 0.87, with a standard deviation of 0.04. In the present investigation upon the gill of *Venus*, the R.Q. in sea water of density 1.025 and at the slower shaking rates was almost 0.87, with a standard deviation of 0.05.

DISCUSSION

From the results of this investigation it seems probable that factors other than ciliary activity account for the stimulating effect of diluted sea water upon the respiration of the clam's gill or mantle. The experimental results presented here do not shed much light upon the nature of this effect or of the action of salt solutions on oxygen consumption. They serve, however, to confirm and extend some observations by others upon different species. The respiratory responses of the gill in *V. mercenaria* are more permanent and predictable than are those in *M. edulis*, the only other Lamellibranch in which they have been investigated under the conditions mentioned. Additional studies upon the clam are in progress to determine the effects of varying the ionic composition of the medium.

The observation that chloretone, in concentrations which partially inhibit ciliary activity, increases the O_2-consumption of clam gill (Table 2) lends emphasis to the fact that narcosis of cell functions does not necessarily depend upon a depression of respiration. Warburg (1910) was the first to stress this point, as a result of his experiments upon sea-urchin eggs, in which he found that cleavage and mitosis were inhibited by phenyl urethane in sea water, at a concentration which did not appreciably reduce respiration. Similar relations have since been reported by others. In investigations upon *V. mercenaria*, Navez *et al.* (1941) found that ethyl urethane caused an increase in the O_2 metabolism of the heart up to 20 per cent.

The importance of osmoregulation, as a respiratory determining factor in aquatic animals, is seen in those homoiosmotic species which are normally subjected to changes in salinity, as in the mouths of rivers or in estuaries. There are, however, a few marine or brackish-water forms, generally regarded as poikilosmotic, in which respiration is found to be greater in a hypotonic medium, notably in *Gunda ulvae* (Beadle, 1931) and in *M. edulis* (Bauxin, 1931).

There may be other factors besides osmoregulation involved in the increased respiration of isolated organs in a diluted medium—perhaps cell hydration or a disturbance of intra-cellular ionic balance. If the hypotonic stimulation of respiration which occurs in excised gills of marine invertebrates (*Carcinus*, *Mytilus*, *Venus*) is directly related to an osmotic difference existing in the intact animal between the body cavities and the external medium, it would require the assumption of an irreversibly polarized membrane, since both surfaces are exposed to one solution. The osmotic pressure of the body fluids in most marine invertebrates is equal to, or only slightly greater than, that of sea water. In many of these, however, there is an unequal distribution of ions between the body fluids and the external medium. Such a condition would also involve energy ex-

penditure for maintaining ionic concentration gradients across membranes; and this might account for some respiratory differences in tissues at various salinities, if a membrane polarity exists. It is known, too, that cells have developed powers of differentially absorbing ions, particularly those of potassium and phosphate, while excluding others, such as chloride. Hence one would expect that these states of dynamic equilibrium would be disturbed by dilution or by changing the balance of ions in the medium. On the basis of our present knowledge, however, it seems probable that in isolated tissues there is active regulation of osmotic pressure within the cells and of interstitial fluid.

Limiting the discussion to the conditions in the Lamellibranchs, it should be noted that *M. edulis* has been found to be in osmotic equilibrium with the environment, when adapted to sea water or brackish water down to the lowest limit compatible with life (Conklin and Krogh, 1938). These investigators also showed that, although the saline composition of the body fluids was not different from that in the environment, the total base, or cation, concentration in muscle was less than half that of the sea-water medium. The difference is osmotic pressure was probably made up by organic substances of low molecular weight (see Krogh, 1939). *Mya arenaria* is also known to be a poikilosmotic species (Schlieper, 1929). As regards conditions in *V. mercenaria*, it was found by Cole (1940) that in Maine clams the mantle fluid showed the same depression of the freezing point as did sea water ($\Delta = 1°.76$). For clams living in Delaware Bay water, at $\Delta = 1°.336$, the values were $1°.369$ for mantle fluid and $1°.386$ for blood. A hypertonicity of the body fluids is here indicated, corresponding to about 0.5 atm. osmotic pressure in a medium 24 per cent less concentrated than Maine sea water. He found, too, that there was a uniform distribution of ions between the internal and the external media. In fresh-water Lamellibranchs (and other invertebrates) a definite hypertonicity of the body fluids is maintained in a fresh-water environment. The osmotic pressure of the blood in *Anodonta*, living in fresh water, is equivalent to a freezing point of about $-0°.1$, according to several investigators (see Duval, 1925; Florkin and Duchateau, 1948). In saline solutions, at increasing concentrations above this level, the body fluids of *Anodonta* come into osmotic equilibrium with the medium. Philippson, Hannevart, and Thieren (1910) found that, under these conditions, the osmotic pressure of the body extracts, by the freezing-point method, was somewhat higher than that of the medium, whereas the concentration of electrolytes, determined by electrical conductivity, was distinctly lower. They attributed the difference between the total osmotic pressure and that of the inorganic salts as due to organic substances. It is difficult to draw any conclusions as to the relation between tissue respiration and osmotic conditions in bivalve mollusks from the above results. The clam, *Venus*, seems to have some power of osmotic regulation, unlike *Mytilus;* and it is noteworthy that the respiratory response of clam gill in dilute sea water is more sustained.

Little is known regarding the respiration of the simpler fresh-water invertebrates at different salinities. Palmhert (1933) showed that *Pelmatohydra*, living within a narrow concentration range (fresh water and very dilute sea water) had a constant respiratory rate; but in a higher or a lower concentration (distilled water) the rate fell off, and signs of abnormality appeared. The respiration of *Planaria* was found by Buchanan (1931)

to be depressed by distilled water. Duryee (1932) showed that in *Planaria* and amphibian embryos the O_2-consumption was reduced in distilled water and increased in Ringer's solution, as compared with tap water. Hayes (1930) observed a decline in rate of O_2-consumption in *Paramaecium caudatum* when brought into water of increasing salt concentration up to 4 per cent sea water, and an increase in rate in stronger solutions.

Certain observations suggests that the body fluids of some animals in greatly diluted waters undergo qualitative changes unlike those occurring during transfer from sea to brackish water. The retention of organic matter to compensate for loss of salts in *Anodonta* (Philippson *et al.*, 1910) is a case in point. In addition to reduced osmotic pressure, there may be a depletion of trace elements below their effective concentrations. Schlieper (1931) found that the respiration of *Gammarus locusta* was almost constant in different concentrations of brackish water but increased in very dilute solutions. According to a determination made by Bateman (1933), the blood of *Gammarus* is hypertonic in brackish water; and hence he believes that Schlieper's results on respiration—indicating osmotic regulation only at great dilution—may require a different interpretation.

Some other factor is probably also involved in the respiratory effects of isotonic salts upon tissues. An unbalanced condition of the ions of sea water would be produced by adding isotonic NaCl solution to it. An extreme case, illustrating ionic imbalance, is seen in the experiments of Pieh (1936), in which she found that the swelling of *Mytilus* gill in pure isotonic NaCl solution was reduced about half on adding sea water to 0.2 per cent, and almost completely in mixtures containing 1 per cent or more of sea water. A comparable relationship obtains in the respiration of the clam's gill: the increased rate observed in isotonic NaCl solution was abolished by adding less than an equal amount of sea water to it (Table 3). One would ordinarily attribute this effect to the antagonism between Na and Ca ions. But, since isotonic NaCl containing 0.1 per cent $CaCl_2$ produced less inhibition than did the NaCl–sea-water mixture containing less than half as much calcium, it is probable that magnesium in the sea water supplemented its action.

The influence of calcium in low concentrations in offsetting the harmful effects of pure water was pointed out by Weil and Pantin (1931), working with *Gunda ulvae*. These worms were found to live better (undergo less swelling) in stream water containing considerable $CaCO_3$ than in Plymouth tap water, which was poor in calcium. The addition of $CaCl_2$ to distilled or tap water had a beneficial effect on survival, probably by reducing the permeability of the body to water.

The inhibitory action of HCN upon respiration has been utilized to investigate the relation between osmotic regulation and metabolism in homoiosmotic animals. The interpretation of its effects upon cells and tissues in various solutions seems to be more limited because the criteria for work performance, osmotic or otherwise, are often obscure. In the Protozoa, which usually possess contractile vacuoles, the problem is simplified but is still complex. Thus, in certain freshwater Peritricha, Kitching (1938) found that cyanide reduced the output of the contractile vacuoles and led to swelling of the body. On returning the animals to cyanide-free water, he observed a supernormal rise in output of the vacuoles—apparently compensatory—and an initial shrinking of the body; then a gradual reduction in the vacuolar output to the

original rate, further shrinkage to less than normal size, followed by a return to normal body volume. In primitive Metazoa the intra-cellular accumulation of fluid has also been noted. In *Gunda*, which lacks a true body cavity, Beadle found (1931, 1934) that, when it was placed in fresh water, there was swelling of the parenchyma and finally the appearance of fluid as vacuoles in endoderm cells. Whatever the route of water elimination may be—whether by discharge into the digestive tract or through the protonephridia, as Krogh (1939) believes—one cannot overlook the importance of intra-cellular changes in the regulatory process. It was found that anoxia or exposure to cyanide caused greater swelling of these animals. In each of the above cases, regulation of the water content of the body was attributed to osmotic processes in the cells, dependent upon metabolism. Intra-cellular osmotic processes are less obvious in higher animals than in those primitive forms in which swelling and vacuole formation occur, although probably well developed in organs of absorption and excretion. In aquatic invertebrates surface epithelial cells (of gill, mantle, etc.), as well as those of renal and digestive tubes, may be differentiated to perform special functions, while at the same time maintaining a cell-osmotic equilibrium.

SUMMARY

A preliminary investigation of the conditions responsible for stimulation of respiration in excised tissues of the clam *V. mercenaria* by dilute sea water and by isotonic salt solutions has been made.

The rate of oxygen consumption of gill tissue was found to be inversely proportional to the density of the sea water between 1.012 and 1.030 for clams living in sea water of density 1.024. The rate of respiration in sea water 1.015 was more than 40 per cent higher for gill, and 12 per cent higher for mantle tissue, than in sea water of density 1.025; it was 15 per cent lower for muscle sections.

The ciliary activity of the gill in sea water at this dilution was definitely lower than in natural sea water, so that this can be eliminated as a factor in the increased respiration. An analogy between the effect of dilute sea water (1.015) and that of chloretone (0.1 per cent) is seen, in that, under each of these conditions, the O_2-consumption was increased, while at the same time ciliary activity was retarded.

An increase of 47 per cent in the O_2-consumption of gill was observed in isotonic NaCl solution. This was largely suppressed when $CaCl_2$ was present in the solution at the same concentration as in sea water. In a mixture of isotonic NaCl with sea water, in a 3:2 or a 1:1 ratio, there was no increase in the respiratory rate over that in sea water.

Cyanide (M/1,000) was without effect upon the O_2-consumption of the gill during July and August, but it produced moderate inhibition during the autumn months. It brought about inhibition of about 60 per cent in mantle and muscle tissues. The augmented respiration of gill and mantle in diluted sea water was inhibited relatively more by cyanide than it was in natural sea water.

The average respiratory quotient of the gill was found to be 0.87—, when measured in sea water of density 1.025, and 0.94 in sea water 1.015.

On the basis of observations by other investigators upon Lamellibranchs, it seems probable that *V. mercenaria* possesses more power of osmotic regulation than do other species; and this may be reflected in the respiratory responses of certain excised tissues in dilute sea water.

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CHANGES IN THE CARBOHYDRATE AND FAT CONTENT OF THE JAPANESE BEETLE (POPILLIA JAPONICA NEWMAN) DURING METAMORPHOSIS

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THE physical and chemical aspects of metamorphosis in the Japanese beetle have been studied in this laboratory over a period of many years. Ludwig (1931) determined changes in weight, water content, and respiratory metabolism during the metamorphosis of the third-instar larva to the adult. In this process there is a loss of weight amounting to half the maximal larval weight; and, at emergence, the loss amounts to one-third the pupal weight. This reduction is largely the result of a loss of water, the water content decreasing from approximately 78 per cent in the larva to 67 per cent in the newly emerged adult. The rate of respiratory exchange follows the U-shaped curve which has been found by many workers to be characteristic of the metamorphosis of holometabolous insects. Ludwig (1934) found that during metamorphosis the pH of the blood changes from an average of 7.07 in the larva to 6.79 in the late prepupa. Pupation is accompanied by a change to approximately 6.95, and it remains relatively constant at this value throughout the pupal stage. It was suggested that the increased acidity of the late prepupa is the result of autolytic changes which are evident in this stage.

Anderson (1948) made an analysis of the nitrogenous components and determined changes in the distribution of nitrogen during the metamorphosis of the Japanese beetle. He found that the total nitrogen content does not change but that the distribution of nitrogen varies as follows: (1) at pupation there is a large decrease in the percentage of total nitrogen present in the water-insoluble compounds, followed by a gradual increase in this fraction during the remainder of pupal life; (2) an increase in amino acid nitrogen occurs at pupation and is followed by a decrease in this fraction during the remainder of the pupal stage; (3) the nitrogen present in the soluble protein (proteose and peptone) decreases significantly between the larval and the early prepupal stages, rises to a high value on the second to third day of pupal life, and then returns to its former low level by the sixth to seventh day of the pupal stage. These complementary shifts in the distribution of nitrogen are be-

lieved to indicate the destruction of larval tissues in the early pupa, followed by the gradual construction of the definitive structures of the adult during the remainder of the pupal stage. Anderson found that the breakdown of insoluble proteins does not coincide with the decrease in oxygen consumption or the increase in the acidity of the blood but occurs later in metamorphosis. Hence he believed that these changes condition the insect so that the degradation of larval proteins can occur. The increase in acidity would thus be a causative factor rather than the result of the autolysis of larval tissues.

The purpose of the present study is to determine changes in glucose, glycogen, and fat during metamorphosis. These compounds are believed to furnish energy for the insect. Since no food is ingested during its transformation from larva to adult, it must obtain energy from the oxidation of materials already stored by the larva. A study of the changes in these compounds should be of value for an interpretation of the chemical mechanisms involved in metamorphosis. A preliminary report of the changes in glucose content was given by Ludwig (1932). This work was recently repeated and extended. In addition, an extended series of determinations was made on the changes in amounts and concentration of glycogen and fats. These results are integrated with those reported in the earlier studies.

MATERIAL AND METHODS

Japanese beetle larvae were brought into the laboratory and reared in individual 1-ounce metal salve boxes containing moist soil, to which several grains of wheat were added to serve as food. All experimental animals were kept at a temperature of 25° C. They were examined every 5 or 6 days and kept supplied with wheat grains throughout the larval stage. When an individual was recognized as an early prepupa (Ludwig, 1931), it was placed in a separate group and examined daily. The date on which the insect reached a particular stage in metamorphosis was recorded on each box. In this manner carefully timed records were obtained for each experimental animal. Readings on glucose, glycogen, and fat were made on groups of early prepupae, late prepupae, pupae on each day of pupal life, and adults within 24 hours of emergence.

Determinations of reducing substances, expressed as glucose, were made by the Hagedorn and Jensen procedure (see Hawk, Oser, and Summerson, 1947, p. 528). Analyses were made on entire animals by the following modification of this procedure. The animal, after it was weighed, was thoroughly crushed in a test tube containing the $NaOH–ZnSO_4$ solution and heated for 3 minutes in boiling water to complete the precipitation of proteins. The solution was then filtered through washed cotton. Five cc. of the $K_3Fe(CN)_6$ solution were added to the filtrate, which was then placed in a boiling water-bath for 15 minutes. If the solution became colorless, 5 cc. more of the ferricyanide were added, and the solution returned to the boiling water-bath for another 15-minute period. This process was repeated until the yellow color of the ferricyanide remained after the period of boiling. The solution was then allowed to cool, after which 3 cc. of the iodide–sulphate chloride solution were added for each 2 cc. of the ferricyanide already added. It was then acidified, with 1 cc. of 3 per cent acetic acid for each cubic centimeter of ferricyanide, and was titrated with standardized $Na_2S_2O_3$, using a starch solution as an indicator. Results were obtained as milligrams of glucose per individual.

Glycogen and fat determinations were made on desiccated insects. When the insect had reached the desired stage of

metamorphosis, it was weighed and dried to a constant weight under vacuum desiccation. Water contents were calculated from the weights before and after complete desiccation. Glycogen was precipitated by a modified Pflüger technique (Good, Kramer, and Somogyi, 1933). The glycogen was then hydrolyzed to glucose by boiling it in 0.6 N HCl for 3 hours in a water-bath. The amount of glucose thus formed was measured by the procedure outlined above, and this value was multiplied by 0.93, the glycogen conversion factor, to obtain milligrams of glycogen.

Determinations of fat were made by ether extraction in a Soxhlet apparatus with anaesthesia ether, previously filtered through a Soxhlet thimble. The dried insects were pulverized with specially purified sand (see Bloor, 1929, p. 282) and transferred to the Soxhlet apparatus. Extractions were continued until no further residue was obtained upon the evaporation of the ether. After extraction the ether containing the dissolved fat was poured into a weighed beaker, which had been cleaned and then dried over anhydrous $CaCl_2$. The beaker was then covered with filter paper to keep out dust and kept at room temperature until the ether had completely evaporated. It was then placed in a desiccator for several hours before it was weighed again. Beakers containing extracted fat were kept in desiccators for several days before their final weights were determined. The difference between the final and the initial weights of the beaker was the milligrams of fat from the extracted insects. This procedure gave values for the free, or ether-extractable, fat. Fat extractions were made on groups of 5 insects, whereas measurements of reducing substances (glucose) and glycogen were made on individual insects.

TABLE 1

CHANGES IN THE GLUCOSE CONTENT OF THE JAPANESE BEETLE DURING METAMORPHOSIS

Stage of Development	No. of Insects	Av. Weight (Mg.)	Av. Weight of Glucose (Mg.)	Glucose as Per Cent Wet Weight
Third-instar larva	44	239.6	1.12	0.52±0.011
Early prepupa	25	237.7	0.99	0.42±0.011
Late prepupa	20	223.5	1.14	0.52±0.015
Pupa:				
Just molted	25	202.6	1.57	0.79±0.023
1-day	15	200.8	1.38	0.68±0.017
2-day	16	207.3	1.40	0.68±0.021
3-day	14	208.1	1.45	0.69±0.013
4-day	15	211.0	1.59	0.75±0.017
5-day	15	233.5	1.77	0.76±0.014
6-day	16	206.1	1.56	0.76±0.014
7-day	14	198.7	1.64	0.80±0.015
8-day	14	215.2	1.80	0.83±0.023
9-day	15	208.2	1.79	0.86±0.014
Adult, just molted	19	133.0	1.31	1.00±0.029

OBSERVATIONS

The results of the glucose determinations are given in Table 1 and Figure 1. Since some adults emerged on the tenth day following pupation at 25° C., determinations on pupae were limited to the

first 9 days of pupal life. The table contains the average weights of the insects, the average weight of the glucose, and the average percentage of glucose per individual. There is a large variation in the weights of different insects at the same stage of metamorphosis. Since the amount of glucose is greater in the larger insects, the percentage values at different stages are considered more comparable than the weights of the substance. In Table 2 is given a statistical comparison of these values for different stages. The change from the third-instar larva to the early prepupa is accompanied by a small but significant change in the amount of glucose present, the percentage value dropping from 0.52 to 0.42. The glucose then rises to its former value in the late prepupa and reaches 0.79 per cent in the early pupal stage. This increase represents a synthesis of glucose, since the amount of glucose increases even though this period of metamorphosis is accompanied by a decrease in the weight of the insects. During the first day of pupal life, there is a small but significant drop to 0.68 per cent. It remains constant for about 2 days and then increases slowly throughout the remainder of the pupal stage, reaching a value of 0.86 per cent in the 9-day pupa. The emergence of the adult is accompanied by a decrease in the amount of glucose; but, since there is a loss of one-third the pupal weight during emergence, glucose rises to a high value of 1.0 per cent in the newly emerged adult.

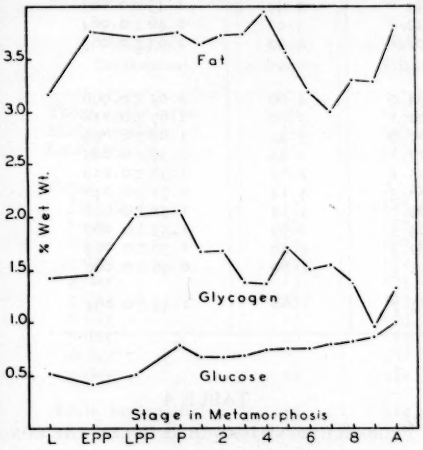

FIG. 1.—Changes in the concentration of glucose, glycogen, and fat during metamorphosis. Abbreviations: *L*, larvae; *EPP*, early prepupae; *LPP*, late prepupae; *P*, pupae; *A*, adults. Numbers after *P* indicate age of pupae.

TABLE 2

PROBABLE SIGNIFICANCE BETWEEN CONCENTRATIONS OF GLUCOSE AT DIFFERENT STAGES OF METAMORPHOSIS

Stages Compared	Difference between Means	Difference/ P.E. of Difference
Third-instar larva and early prepupa	0.10	6.6
Early and late prepupa	0.10	5.5
Late prepupa and pupa, just molted	0.27	10.0
Pupa:		
Just molted and 1-day	0.11	3.9
3- and 4-day	0.06	2.8
3- and 5-day	0.07	3.7
6- and 7-day	0.04	2.0
6- and 9-day	0.10	5.2
Adult and 9-day pupa	0.14	5.8

The values obtained in the glycogen analyses are given in Table 3 and in Figure 1, and a statistical comparison of the glycogen, expressed as percentage wet weight, is contained in Table 4. From these data it is observed that the glycogen content, both as percentage body weight and as milligrams per individual, increases from the third-instar larva to the early pupa, following which it decreases irregularly during the pupal stage. The increase in percentage of glycogen from the third-instar larva to the early pupa (1.43–2.07) is statistically significant. The decrease from the late

prepupal or early pupal stage to the 4-day pupa (2.03 or 2.07 to 1.38) is also significant. Between the fourth and fifth days of pupal life, there is a synthesis of glycogen, since it increases in amount and its percentage concentration shows a significant increase (1.38–1.71). Following the remainder of the pupal stage. Emergence of the adult is associated with a loss of fat from an average of 5.73 mg. in the 9-day pupa to 4.36 mg. in the newly emerged adult. However, the percentage value increases to 3.82 in the adult.

TABLE 3

CHANGES IN THE GLYCOGEN CONTENT OF THE JAPANESE BEETLE DURING METAMORPHOSIS

Stage of Development	No. of Insects	Av. Weight (Mg.)	Av. Weight of Glycogen (Mg.)	Glycogen as Per Cent Wet Weight
Third-instar larva	17	244.6	3.34	1.43±0.103
Early prepupa	13	239.6	3.42	1.46±0.064
Late prepupa	15	208.8	4.23	2.03±0.065
Pupa:				
Just molted	11	194.9	4.00	2.07±0.096
1-day	13	202.1	3.19	1.67±0.118
2-day	15	207.0	3.43	1.67±0.099
3-day	12	177.1	2.43	1.39±0.085
4-day	12	195.2	2.67	1.38±0.058
5-day	13	188.3	4.14	1.71±0.046
6-day	13	209.0	3.14	1.51±0.090
7-day	11	193.5	2.99	1.55±0.080
8-day	14	189.3	2.48	1.38±0.093
9-day	15	165.1	1.60	0.96±0.069
Adult, just molted	12	123.7	1.68	1.33±0.094

lowing this increase, there is a steady decrease in glycogen during the remainder of the pupal stage, its percentage value being 0.96 in the 9-day pupa. No change is associated with the emergence of the adult, but the percentage value rises because of the weight loss which accompanies this transformation.

Changes in the concentration of fats are shown in Table 5 and in Figure 1. The percentage of fat increases from 3.16 in the third-instar larva to 3.76 in the early prepupa. It remains relatively constant near this figure until the fifth day of the pupal stage, when it shows the beginning of a sharp decrease. This decrease continues during the next 2 days, the fat reaching a low level, ranging from 3.0 to 3.3 per cent, where it remains dur-

TABLE 4

PROBABLE SIGNIFICANCE BETWEEN THE CONCENTRATION OF GLYCOGEN AT DIFFERENT STAGES OF METAMORPHOSIS

Stages Compared	Difference between Means	Difference/ P.E. of Difference
Third-instar larva and late prepupa	0.60	5.0
Late prepupa and 1-day pupa	0.36	2.7
Late prepupa and 4-day pupa	0.65	7.2
Pupa:		
4- and 5-day	0.33	4.7
5- and 8-day	0.33	3.2
5- and 9-day	0.75	9.4
8- and 9-day	0.42	3.7
Adult and 9-day pupa	0.37	3.2

Table 5 also contains figures showing the water content of the different stages. The water content of the larva was 74.5 per cent. It increased to 77.1 per cent in the early pupa, remained relatively constant during the pupal stage, and decreased to 69.2 per cent during emergence.

A comparison of the analyses of third-instar larvae and newly emerged adults is shown in Table 6. The weights of larvae and adults are the average values for the three series of insects used in these experiments. The value given for the feces contained in the larvae was obtained from Ludwig (1946). No values are included for protein or other nitrogenous compounds, since Anderson (1948) found that no significant change in nitrogen occurs during the metamorphosis of the Japanese beetle. The table shows that the weight lost during metamorphosis is the result of a loss of water and feces and of the utilization of glycogen and fat.

TABLE 5

CHANGES IN THE FAT CONTENT OF THE JAPANESE BEETLE DURING METAMORPHOSIS

Stage of Development	No. of Insects	Av. Weight (Mg.)	Av. Water Content (Per Cent)	Av. Weight of Fat (Mg.)	Fat as Per Cent Wet Weight
Third-instar larva	30	212.2	74.5	6.71	3.16
Early prepupa	15	206.5	76.9	7.76	3.76
Late prepupa	15	185.4	77.4	6.88	3.71
Pupa:					
Just molted	15	175.0	77.1	6.56	3.75
1-day	29	172.6	78.3	6.28	3.64
2-day	15	188.2	76.8	7.03	3.73
3-day	15	172.3	77.8	6.45	3.74
4-day	15	185.6	78.1	7.34	3.95
5-day	15	173.0	77.6	6.16	3.56
6-day	15	183.5	78.6	5.93	3.23
7-day	26	174.2	78.6	5.20	3.00
8-day	25	187.1	77.8	6.17	3.30
9-day	15	174.5	78.4	5.73	3.28
Adult, just molted	15	114.0	69.2	4.36	3.82

TABLE 6

CHANGES IN WEIGHT DURING METAMORPHOSIS

	Larva		Adult		Weight Lost (Mg.)
	Per Cent	Weight (Mg.)	Per Cent	Weight (Mg.)	
Observed	.....	232.1	.....	123.6	108.5
Water	74.5	172.91	69.2	85.53	87.38
Feces	7.4	17.17	.....	.....	17.17
Glucose	0.52	1.21	1.00	1.23	.....
Glycogen	1.43	3.32	1.33	1.64	1.68
Fat	3.16	7.33	3.82	4.72	2.61
Total (calculated)	.....	.....	.....	.....	108.84

DISCUSSION

Tables 1, 3, and 5 show that an increase in the percentage values of glucose, glycogen, and fat occurs during the change from the third-instar larva to the early pupa. However, this transformation is associated with a loss of weight, due largely to the elimination of feces and to a loss of water. Because of this weight loss, the percentage of the various solids of the insect is increased. An examination of Tables 1 and 3 will show that there is an actual increase in the weights of the glucose and glycogen during this phase of metamorphosis. Anderson (1948) found a sharp decrease in the insoluble proteins and a corresponding increase in the soluble nitrogen fractions at the time of pupation. Hence there is the possibility that some of the products which result from the degradation of the insoluble proteins may be changed to carbohydrates, resulting in the increases just described. Anderson reported a gradual increase in the insoluble protein during the remainder of the pupal stage. Since the glucose and glycogen both decrease during the next few days of the pupal period, they may be partly used in the resynthesis of the insoluble protein.

The losses of weight and water described in the present paper are essentially the same as reported in the previous studies (Ludwig, 1931). However, there is one outstanding difference. In the previous work the water content of the third-instar larva was given as 78.1, and of the early pupa as 74.2 per cent, whereas in the present studies these values are 74.5 and 77.1 per cent, respectively. Hence, the change from the larva to the pupa resulted in the loss of less water in the present experiments than in the earlier work. Ludwig (1946), in his studies on the effect of DDT on the metabolism of the Japanese beetle, made the observation that larvae collected in different localities may have different water contents. He used one group of larvae with an initial water content of 72.8 and another group with 77.6 per cent. The larvae used in the present studies were obtained from the same locality as those with the low water content used in 1946.

The figures contained in Table 6 indicate no difference in the weight of glucose in larva and adult. However, in the change from larva to prepupa, from prepupa to newly molted pupa, and during the pupal stage, there are changes in both the weights and the concentration of glucose, as already described. These changes are essentially the same as those reported by Crescitelli and Taylor (1935) for the bee moth, *Galleria mellonella*, except that their values are lower than those given here for the Japanese beetle. Frew (1929) observed a marked increase in glucose during the pupal stage of the blowfly (species not given), and Courtois-Drilhon (1931) reported an increase in the glucose content of the early pupae of three species of Lepidoptera (*Attacus pernyi*, *Sphinx ligustri*, and *Saturnia pyri*). This increase continued from the time of pupation until December and was followed by a steady decrease until emergence the following April. Evans (1932) observed that the glucose content of the blowfly, *Lucilia sericata*, falls steadily during histolysis and then remains fairly constant during histogenesis. In 1934, he studied the chemical changes associated with metamorphosis of the mealworm, *Tenebrio molitor*, and found a steady decrease in glucose throughout metamorphosis. Hence glucose may serve as a source of energy during the pupal stage of some species.

Many investigators have studied the utilization of glycogen during the process of metamorphosis in insects. Bataillon (1893), Vaney and Maignon (1905), and

Kotake and Sera (1909) showed an irregular but progressive decrease in glycogen during the pupal stage of the silkworm, *Bombyx mori*. Similar results were obtained by Weinland (1907) for the blowfly, *Calliphora vomitaria;* by Strauss (1911) for the honeybee, *Apis mellifica;* by Rudolfs (1926) for the tent caterpillar, *Malacosoma americana;* and by Courtois-Drilhon (1931) for the three species of Lepidoptera referred to above. Frew (1929) stated that he found no glycogen in the blowfly, and Evans (1932 and 1934) found a steady decrease in the total carbohydrate content of the blowfly, *L. sericata*, and of the mealworm, *T. molitor*. However, he did not give values on the quantity of glycogen present in these forms. The present study shows an irregular but rapid decrease in glycogen during the first 4 days of pupal life, an increase on the fifth day, and a decrease throughout the remainder of the pupal stage. These results were obtained in each of three separate groups of experiments.

The increase in glycogen on the fifth day of the pupal stage coincides with a decrease in fat and suggests that glycogen is synthesized from fat. Furthermore, the decrease in weight of the fat is sufficient to account for the observed increase in weight of glycogen. It appears likely that glycogen is used as the source of energy during the pupal stage, and, when it reaches a low level, it is replenished at the expense of stored fats. Couvreur (1895), Vaney and Maignon (1905), and Kotake and Sera (1909) observed a decrease in the fat content of the silkworm in the prepupal period. This is the period during which glycogen is being synthesized, and the glycogen reaches a maximal value at the time that the pupa is formed. Couvreur suggested that, since the increase in glycogen coincides with a decrease in fat, the glycogen may be formed from fat. Kotake and Sera found no rise in glycogen after the feeding stage. They denied any conversion of fat into glycogen. Weinland (1907) also observed an increase in carbohydrate during the pupal stage of the blowfly, *C. vomitaria*, and suggested that protein may be the source of the carbohydrate. In the Japanese beetle there is no loss of protein during the pupal stage. Hence the glycogen could not be synthesized from protein in this insect. Its only possible source appears to be from stored fat. Frew (1929) found that in the blowfly there is no change in the fat content during the first part of the pupal stage. During the latter half of this stage the fat content continually decreases. Frew also suggested that fat may be used in the formation of carbohydrate.

Fat may possibly be used as a source of energy on emergence, as indicated by the decrease in amount of fat which occurs at that time. However, it is also likely that some of the ether-extractable materials may be used in the formation of the epicuticle of the adult. The lipoid constituents of the epicuticle are not present in the free state (Wigglesworth, 1939) and therefore would not be extracted with ether. Evans (1934) observed that in the mealworm, *T. molitor*, fat is consumed at the beginning and at the end of metamorphosis, very little being utilized during the middle of the period, when metabolism is lowest. In the blowfly, *L. sericata*, Evans (1932) found a rapid decrease in fatty acid content until the eighth day, when it decreased very slowly. A definite synthesis of fatty acid then occurred, and a second maximum was reached on the fourteenth day. In general, other work on the ether-extractable fats shows a general decrease during the period of metamorphosis. This work is reviewed by Needham (1931 and 1942).

The possible conversion of fat to glycogen would explain the low respiratory quotients (ranging from 0.4 to 0.7) observed during the pupal stage of the Japanese beetle (Ludwig, 1931). However, the time for this carbohydrate synthesis appears to be during the middle of the pupal stage. A re-examination of the data collected during the respiratory studies failed to show a very low quotient at that time. Low respiratory quotients (averaging between 0.52 and 0.68) were obtained throughout the pupal period. Since the present study indicates that glycogen supplies the energy required during this stage, there seems to be no definite correlation between the respiratory quotient and the type of metabolism occurring in the insect. Taylor and Steinbach (1931) give a discussion of the respiratory quotient and its importance during pupal metabolism. They point out that the oxidation of fats cannot occur without the oxidation of carbohydrate. Hence it is false to assume that a quotient of 0.69 (the value obtained in their work) represents an exclusive oxidation of fat. In view of the recent work on the catabolic cycles involved in the utilization of carbohydrates, fats, and proteins in tissues, it seems evident that a re-examination of the whole problem of the respiratory quotient and its significance is greatly needed.

SUMMARY

1. Determinations were made on the glucose, glycogen, and fat content of Japanese beetles at the following stages of metamorphosis: third-instar larva, early prepupa, late prepupa, pupae on each day of pupal life, and newly emerged adults.

2. The glucose content of the third-instar larva was 0.52 per cent. It decreased to 0.42 per cent in the early prepupa and then increased to 0.79 per cent in the newly molted pupa. During the pupal stage the glucose dropped to 0.68 per cent in the 1- and 2-day pupae and then began to rise slowly, reaching a high value of 0.86 per cent on the last day of the pupal stage. Emergence was associated with an actual loss of glucose, but, because of the loss of weight which occurs at that time, the percentage value is increased.

3. Glycogen content increases from 1.43 per cent in the larva to 2.07 per cent in the early pupa. During the pupal stage there is a rapid loss of glycogen during the first 4 days, an increase on the fifth day, and a decrease throughout the remainder of pupal life. No significant change in glycogen was observed on emergence, although the percentage value increased because of the weight loss which occurs at that time.

4. Fat content remained at a constant value during the prepupal and first 4 days of the pupal stage. It decreased sharply on the fifth and sixth days and remained relatively constant at a lower level throughout the remainder of the pupal stage. A loss of fat occurs on emergence, probably because of its utilization in the formation of the epicuticle of the adult.

5. These results indicate the utilization of glycogen for energy during the pupal stage. When the glycogen reaches a low value, it is replenished at the expense of stored fat.

6. The increase in glucose and glycogen which is observed at the time of pupation may possibly be the result of a breakdown of insoluble proteins which occurs at that time, the degradation products being temporarily stored as carbohydrates.

7. The low respiratory quotients (average 0.5–0.7) observed during the pupal

stage cannot be used in an interpretation of the type of metabolism occurring at that time, since these quotients suggest that fats are being oxidized and the experiments indicate the utilization of glycogen.

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DEVELOPMENT OF THE GLYCOGEN BODY OF THE CHICK SPINAL CORD

II. EFFECTS OF UNILATERAL AND BILATERAL LEG-BUD EXTIRPATION[1]

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THE normal morphogenesis, vasculogenesis, and anatomical relationships of the "glycogen body" of the chick spinal cord have recently been described in detail by Watterson (1949). This unusual structure develops within the roof plate of the neural tube at the level of spinal nerves 26–29, which are the major roots of the sciatic nerve (see Watterson, 1949, Pl. I). Within this roof plate, at approximately 8 days of incubation, appear special cells which store glycogen intra-cellularly (Terni, 1924; Watterson, 1947). Whether these glycogen-storing cells actually originate within the roof plate or whether they migrate into it from other parts of the neural tube has not yet been determined. Once they are recognizable within the roof plate, they proliferate rapidly and increase in diameter until their total mass constitutes the glycogen body, which lies between and protrudes above the dorsal funiculi. To date, this structure has been described only in birds. The amount of glycogen stored by the glycogen body is considerable; by 14–19 days of incubation, 60–80 per cent of the extracted dry weight of the glycogen body is represented by glycogen, an amount constituting roughly 5–10 per cent of the total body glycogen (Doyle and Watterson, 1949).

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Nothing is known about the causal factors responsible for initiating the development of this derivative of the neural tube and for controlling its size. Since the glycogen body is localized so sharply at the level of the dorsal roots of the sciatic nerve, the obvious preliminary step in the search for these causal factors would be to determine whether the dorsal roots of the sciatic nerve are essential for the normal morphogenesis of the glycogen body. The dorsal roots could be eliminated by extirpation of the neural crests at the level of the future sciatic nerves; such an operation would leave the cord *in situ* and presumably would not disturb the developmental relationships in so far as the ventral half of the cord is concerned; but quite likely the thin roof plate would be injured by the extirpation, as well as that part of the somites immediately adjacent to the dorsal part of the neural tube. In either case the morphogenesis of the glycogen body might well be modified considerably, and yet the effect could not be attributed to the absence of the dorsal roots of the sciatic nerve. An alternative method would be to isolate the future glycogen-body level of the neural tube, scrape off the neural-crest cells, and transplant the neural tube to the coelom of a host embryo. However, Bueker (1943) has stated with reference to the chick embryo (p. 118) that "two-thirds of all spinal cord transplants were very abnormal and had to be discarded." This led him to state that "the spinal cord is a

delicate structure which does not stand up well against the hazards of transplantation." It would appear that this method would introduce even more secondary factors which could not be evaluated.

However, there is a method whereby the size of the dorsal roots, spinal ganglia, dorsal funiculi, and posterior gray columns can be reduced considerably *with little possibility of direct injury to the cord*, viz., by extirpation of the leg buds. Bueker (1947) has demonstrated that radical leg-bud extirpation in the chick embryo (including removal of the most lateral parts of the somites) is capable of reducing (1) the peripheral nerves of the operated side to one-third to one-quarter the size of those located contralaterally, (2) the spinal ganglia on the operated side approximately one-half to two-thirds, (3) the area of the dorsal funiculus by 50–70 per cent, and (4) the area of the posterior column by 37 per cent, etc. Even though this method does not completely eliminate the dorsal roots, it reduces their size so considerably and the size of the white and gray matter associated with them that it should be possible to determine whether these parts of the nervous system exert any influence upon the development of the glycogen body.

The primary purpose of the present paper is twofold: (1) to determine whether the reduction in the size of certain components of the spinal cord and sciatic nerve which can be accomplished by radical leg-bud extirpation has any effect upon the development of the glycogen body and (2) to see whether, if some effect is detectable, the nature of the modification provides any clue to the major causal factors responsible for the development of the glycogen body. The effects of both unilateral and bilateral leg-bud extirpation will be analyzed. A secondary purpose of this paper is to determine whether a reduction in the size and/or a change in the shape of the glycogen-body level of the spinal cord are associated with a reduction in the size of the vertebral canal and/or a change in its shape or whether the latter is relatively independent of the former.

MATERIALS AND METHODS

White Plymouth Rock and Barred Plymouth Rock eggs were used exclusively. These eggs were from the same flocks which provided the material for the study of the development of the glycogen body under normal conditions (Watterson, 1949). The operative procedure for producing unilateral leg-bud extirpations was essentially that described by Bueker (1943). Usually the right leg bud was extirpated, but in some instances the left leg bud was removed. Embryos submitted to extirpation had been incubated 65 hours or less; it is likely that no nerves have entered the hind-limb area at the time of the operation. Only those specimens were sectioned which showed no external traces of leg structure on the operated side. Bilateral leg-bud extirpation was more difficult to accomplish. In many instances the posterior part of the embryo became so fragile after one leg bud had been removed that any tampering with the opposite side was certain to stretch and rupture the blood vessels. Moreover, it became so fragile that the glass needle sometimes failed to cut through the tissue evenly, with the result that the extirpation was frequently not so complete as desired. In order to facilitate removal of the second leg bud, it was found helpful to touch the leg bud lightly with a glass rod to which a thin solution of celloidin was adhering. This coats the region to be extirpated with a thin celloidin "skin." If the rod is repeatedly dipped into the celloidin solution and

then touched to the leg bud, the capillaries within the leg bud suddenly rupture, the blood seems to congeal, and the area to be extirpated can then be removed somewhat more easily and completely. This congealing can be so controlled that it does not involve the somites or neural tube. Again, only those specimens were sectioned which gave no external indication of leg structure on either side.

In order to have some measure of the amount of periphery extirpated, the number of cell bodies in the lateral motor columns was counted in every section through the glycogen-body level. Only those cells were counted in which the nucleus could be seen distinctly. Since these sections were frequently quite thick (see Table 1), these counts were not too accurate, but they give some measure of the hypoplasia of the lateral motor column. Hamburger (1934) has demonstrated for the wing-bud level that this reduction in the number of lateral motor neurons is correlated with the degree of loss of musculature, one being a linear function of the other.

Table 1 lists the sectioned specimens upon which this study is based. In most

TABLE 1

SECTIONED SPECIMENS AND ESTIMATED COMPLETENESS OF LEG-BUD EXTIRPATIONS

Days of Incubation	Operation*	Thickness (In μ)	No. Cells in Lat. Motor Column		Per Cent Reduction of Periphery†	
			Right	Left	Right	Left
10.0	R1	30	311	2,587	88	0
	R2	30	961	2,731	65	0
10.5	B	12	655	451	80	86
12.0	B	15	363	2,118	89	34
	R1	12	203	3,483	94	0
	R2	12	.....	.....	.....	.....
	L1	15	1,872	296	0	84
14.0	B3	12	352	340	89	89
	B5	30	634	488	80	85
	B6	30	323	813	90	75
	R2	30	939	3,179	70	0
	R7	24	1,403	3,800	63	0
	R9	22	.....	.....	.....	.....
14.5	R	24	805	4,076	80	0
15.0	R2	12	997	3,030	67	0
	R3	24	1,251	4,629	73	0
16.0	B3	23	0	0	100	100‡
	B6	12	2,112	297	34	91
	R4	24	374	3,336	89	0
	L2	30	3,450	866	0	75

* R, right leg bud extirpated; L, left leg bud extirpated; B, both leg buds extirpated.

† Since the amount of hypoplasia in the case of bilateral leg-bud extirpations could not be determined by comparing one side of the cord with the other, it was determined by comparing each side of the cord of the operated embryos with an average value derived from the totals of the control sides of the unilateral extirpations and of one side of three unoperated embryos, ages 12, 14, and 16 days. This average count was 3,214 cells.

‡ The lateral motor columns as such are lacking, but there are a few scattered neuron cell bodies (see Pl. I, Figs. 10 and 11).

instances the lumbosacral level of the vertebral column was excised quickly and fixed in Bouin's fluid; these specimens were stained *in toto* with Mayer's alcoholic hydrochloric acid carmine, imbedded in nitrocellulose, and sectioned serially. Two specimens (12.0 R2 and 14.0 R9) were fixed in absolute alcohol, imbedded in nitrocellulose, sectioned, and stained with Ehrlich's haematoxylin and Best's carmine; all these sections were mounted, but not serially. Best's carmine stains the glycogen content of cells pink if the amount of glycogen is small, red if it is large.

Volumes of the glycogen body, adjacent nervous tissue, and vertebral canal at the level of the glycogen body were determined by projecting each section upon the ground glass of the tracing table, as described by Feeney and Watterson (1946), outlining each section on tracing paper, measuring the areas of all tracings with a planimeter, and calculating the volumes from the obtained areas.[3]

OBSERVATIONS

I. VOLUME OF THE GLYCOGEN BODY IN OPERATED EMBRYOS AND IN CONTROLS

In order to determine whether leg-bud extirpation affects the size of the glycogen body, it was necessary to calculate the volume of the glycogen body in the operated embryos and to compare these values with the volume of the glycogen bodies in normal embryos. Watterson (1949) has calculated the volume of the glycogen body in normal embryos at various developmental stages; these values are recorded in columns 3 and 4 of his Table II, and are plotted both on his

TABLE 2

VOLUME OF GLYCOGEN BODIES AND ADJACENT NERVOUS TISSUE AND RATIO OF THE FORMER TO THE LATTER FOLLOWING LEG-BUD EXTIRPATION*

Specimen No.	Vol. Gly. Body ($In.^3 \times 10^{-3}$)	Av. Vol. Gly. Body ($In.^3 \times 10^{-3}$)	Vol. Adj. Nerv. Tiss. ($In.^3 \times 10^{-3}$)	Av. Vol. Adj. Nerv. Tiss. ($In.^3 \times 10^{-3}$)	Ratio $\frac{\text{Vol. Gly. Body}}{\text{Vol. Adj. Nerv. Tiss.}}$	Av. Ratio
10.0 R1	0.0095	0.0071	0.071	0.065	0.13	0.105
10.0 R2	0.0047		0.059		0.08	
10.5 B	0.0043	0.0043	0.033	0.033	0.13	0.13
12.0 B	0.018	0.018	0.077	0.077	0.23	0.23
12.0 R1	0.020	0.024	0.097	0.107	0.21	0.225
12.0 L1	0.028		0.118		0.24	
14.0 B3	0.047	0.069	0.094	0.165	0.50	0.426
14.0 B5	0.106		0.248		0.43	
14.0 B6	0.054		0.153		0.35	
14.0 R2	0.055	0.062	0.148	0.178	0.37	0.355
14.0 R7	0.070		0.208		0.34	
14.5 R	0.085	0.085	0.198	0.198	0.43	0.43
15.0 R2	0.064	0.131	0.198	0.264	0.32	0.46
15.0 R3	0.198		0.331		0.60	
16.0 B3	0.011	0.0115	0.085	0.097	0.13	0.12
16.0 B6	0.012		0.109		0.11	
16.0 R4	0.132	0.119	0.302	0.298	0.44	0.40
16.0 L2	0.106		0.295		0.36	

* Cubic inches are used as the unit of measure of volume rather than cubic millimeters, in order to facilitate direct comparison with the corresponding volumes in normal embryos given by Watterson (1949). To convert these volumes to cubic millimeters, divide each value by 0.0000610 or multiply each value by 16,387.

[3] We are indebted to Mrs. Helen Bock for assistance with the tracings and measurements and to Mr. John Dunkel for assistance with the sectioning. The photomicrographs were prepared by Mr. Kenji Toda.

Figure 3 and in Figure 1, *A*, of this paper (*solid line*). The volume of the glycogen body in operated embryos was calculated for various developmental stages; these values are recorded in the second and third columns of Table 2 and are plotted on Figure 1, *A*. Growth of the glycogen body following unilateral extirpation is plotted separately (*broken line*) from that following bilateral extirpation (*dotted line*). It will be noted that there are no significant differences in the curves until the sixteenth day of incubation, at which time the volume of the glycogen body of the unilaterals is considerably less than that of the normals, and the volume of the glycogen body of the bilaterals is considerably less than that of the unilaterals.

II. VOLUME OF NERVOUS TISSUE ADJACENT TO GLYCOGEN BODY IN OPERATED EMBRYOS AND CONTROLS

In order to decide whether the total amount of nervous tissue adjacent to the glycogen body is reduced after leg-bud extirpation, it was necessary to calculate the volume of the adjacent nervous tissue in the operated embryos and to compare these values with the volume of the nervous tissue adjacent to the glycogen bodies in normal embryos. Watterson (1949) has also calculated the volume of the nervous tissue adjacent to the gly-

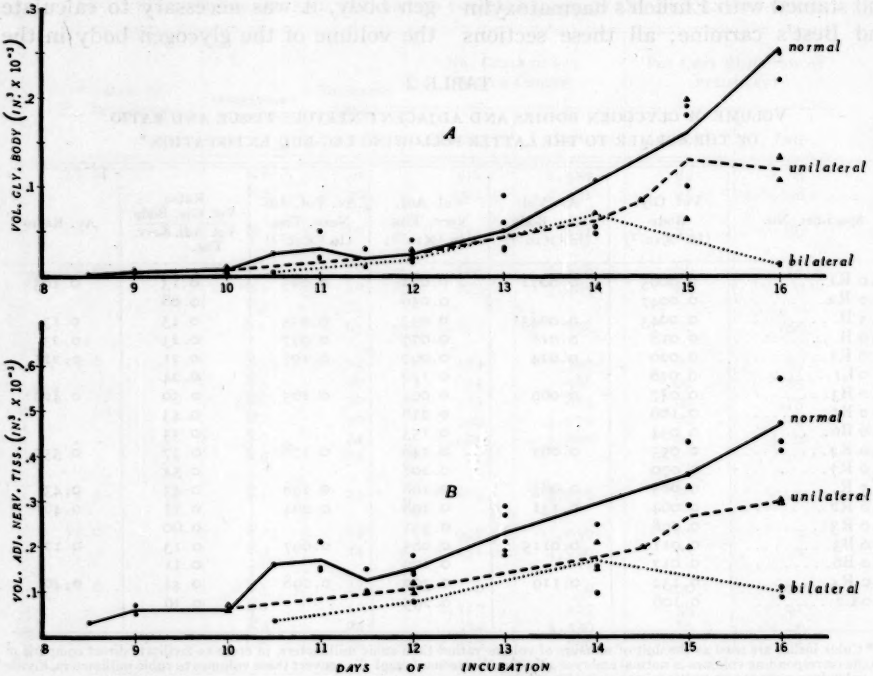

FIG. 1.—*A*, comparison of volume of glycogen body in normal embryos (*solid line*), in embryos submitted to unilateral leg-bud extirpation (*broken line*), and in embryos submitted to bilateral leg-bud extirpation (*dotted line*). *B*, comparison of volume of nervous tissue adjacent to the glycogen body in normal and operated embryos.

cogen body in normal embryos at various developmental stages; these values are recorded in columns 5 and 6 of his Table II and are plotted on his Figure 3 and in Figure 1, *B*, of this paper (*solid line*). The volume of the nervous tissue adjacent to the glycogen body in operated embryos was calculated for various developmental stages, and these values are recorded in the fourth and fifth columns of Table 2; they are plotted on Figure 1, *B*, and, as before, the volumes following unilateral extirpation are plotted separately (*broken line*) from those following bilateral extirpation (*dotted line*). It will be noted that the average volume of this part of the spinal cord is somewhat smaller in operated embryos than in controls at all stages examined; however, the difference between operated and control embryos is very slight until the sixteenth day of incubation, at which time the volume of the nervous tissue adjacent to the glycogen body of the unilaterals is considerably less than that of controls, and the volume of the nervous tissue adjacent to the glycogen body of the bilaterals is considerably less than that of the unilaterals.

VOL. GLYCOGEN BODY

III. THE RATIO VOL. ADJ. NERVOUS TISSUE IN OPERATED EMBRYOS AND IN CONTROLS

The ratio of the volume of the glycogen body to the volume of the adjacent nervous tissue has been calculated by Watterson (1949) in normal embryos at various developmental stages; these values are recorded in columns 7 and 8 of his Table II and are plotted both on his Figure 4 and on Figure 2 of this paper (*solid line*). The above ratio has also been obtained for the operated embryos; these values are recorded in the sixth and seventh columns of Table 2 and, as before, are plotted separately for the unilateral extirpations (*broken line*) and for the bilateral extirpations (*dotted line*) on Figure 2. There are no marked differences between the ratios prior to the sixteenth day of incubation, at which time the ratio is slightly lower than normal in the case of the unilateral extirpations and is considerably lower in the case of the bilateral extirpations.

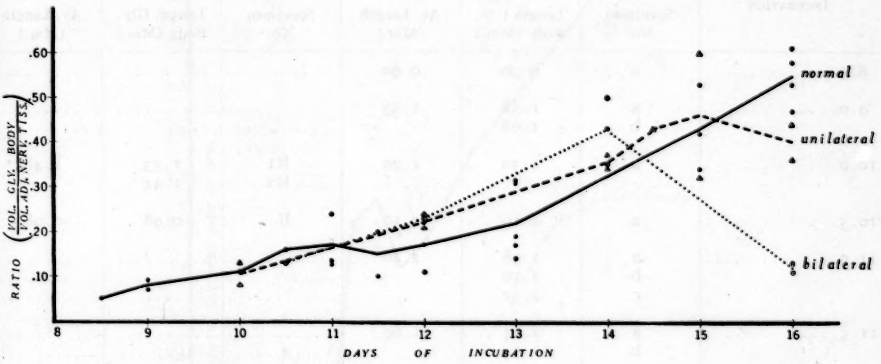

FIG. 2.—Comparison of the ratio of the volume of the glycogen body to the volume of the adjacent nervous tissue in normal and operated embryos.

IV. EFFECT OF EXTIRPATIONS UPON LENGTH AND CROSS-SECTIONAL SHAPE OF GLYCOGEN BODY

In order to determine whether the anteroposterior extent of the glycogen body is modified following leg-bud extirpation, even though the volume remains essentially unchanged in most instances, the length of the glycogen body was measured in the operated embryos. Watterson (1949) has recorded the length of the glycogen body (as number of 60-μ sections) in normal embryos at different developmental stages (see col. 3 of his Table I). These values, converted to millimeters, are recorded in the third and

TABLE 3

LENGTH OF THE GLYCOGEN BODY IN OPERATED AND CONTROL EMBRYOS

Days of Incubation	Controls			Experimentals		
	Specimen No.	Length Gly. Body (Mm.)	Av. Length (Mm.)	Specimen No.	Length Gly. Body (Mm.)	Av. Length (Mm.)
8.5	a	0.90	0.90			
9.0	a	1.38	1.53			
	b	1.68				
10.0	a	1.20	1.20	R1	1.53	1.485
				R2	1.44	
10.5	a	2.10	2.10	B	0.98	0.98
11.0	a	1.98	2.20			
	b	2.10				
	c	2.52				
11.5	a	2.04	1.89			
	b	1.74				
12.0	a	1.68	2.00	B	1.05	1.05
	b	2.10		R1	1.01	1.16
	c	2.22		R2	1.19	
				L1	1.275	
13.0	a	2.16	2.39			
	b	2.10				
	c	2.70				
	d	2.64				
	e	2.34				
14.0				B3	1.31	1.93
				B5	2.64	
				B6	1.83	
				R2	1.77	1.77
				R7	1.78	
				R9	1.76	
14.5				R	1.99	1.99
15.0	a	2.52	2.92	R2	1.74	2.12
	b	3.30		R3	2.50	
	c	2.94				
16.0	a	3.42	3.195	B3	0.782	0.84
	b	3.42		B6	0.900	
	c	2.94		R4	1.90	2.21
	d	3.00		L2	2.52	

fourth columns of Table 3 of this paper and are plotted (*solid line*) in Figure 3. The length of the glycogen body in operated embryos is recorded in the sixth and seventh columns of Table 3, and these values are plotted separately for the unilaterals (*broken line*) and bilaterals (*dotted line*) on Figure 3. There it will be noted that the length of the glycogen body is considerably less after both unilateral and bilateral leg-bud extirpation, except possibly at 10 days of incubation; the value recorded for the 10-day control appears to be unusually small for this stage of development. There is no great difference in the length of the glycogen body in unilaterals, as compared with bilaterals, except at 16 days of incubation, at which time the bilateral leg-bud extirpations are associated with greatly shortened glycogen bodies, and possibly at 10.5 days (see also Fig. 4).

The cross-sectional shape of the glycogen body in normal embryos at various developmental stages has been illustrated extensively by Imhof (1905) and by Watterson (1949), and the changes in cross-sectional shape during normal development are summarized in Figure 2 of the latter paper. In general, the shape of the glycogen body under normal circumstances is quite constant for a given stage of development. This is evident if the right-hand drawings of Figure 4 are examined. Each drawing represents that section in each specimen in which the area of the glycogen body is the greatest. At each of the stages, tracings of the

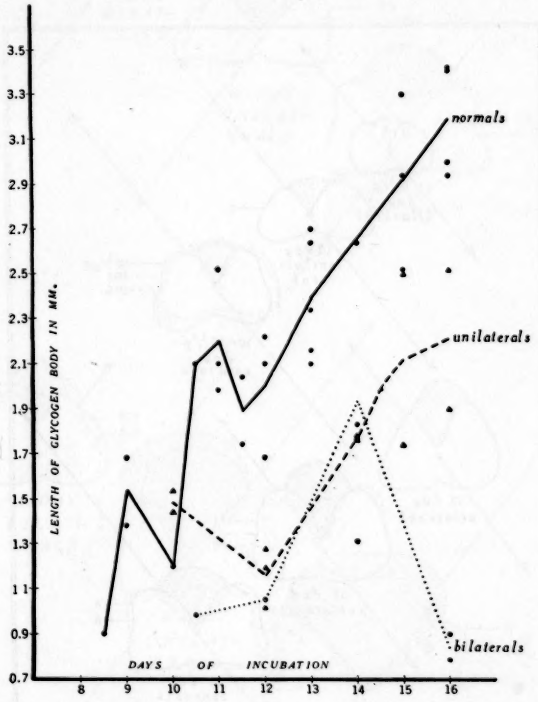

FIG. 3.—Comparison of the length of the glycogen body in normal and operated embryos

glycogen-body level of the spinal cord of two or more control specimens have been superimposed, and the similarity of their cross-sectional shapes is evident. Note particularly the bilateral symmetry of the normal glycogen body. The length of the line running through the neural canal in each of these specimens indicates the average length of the glycogen bodies in the control embryos.

Unilateral leg-bud extirpation causes a definite hypoplasia of the nervous tissue adjacent to the glycogen body on the operated side, such that the spinal cord exhibits a distinct asymmetry (see right side of Pl. I, Fig. 6). This has already

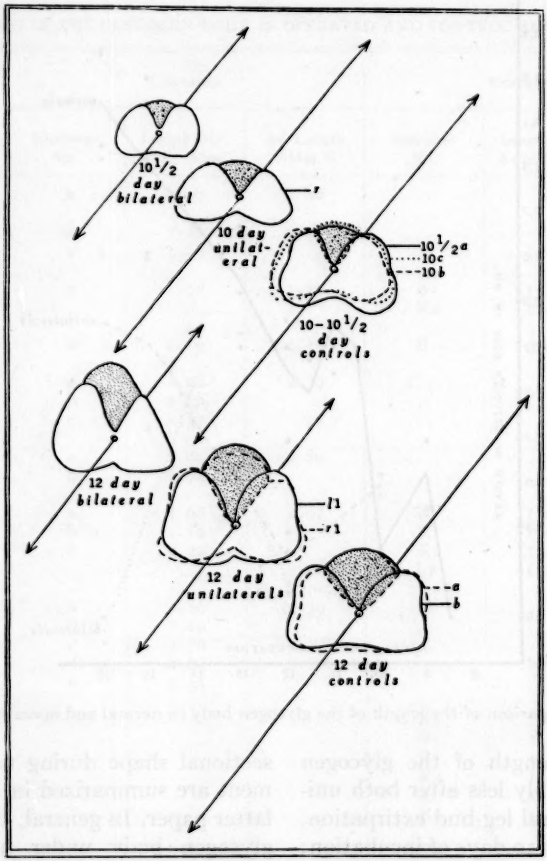

FIG. 4.—Simultaneous comparisons of the length of the glycogen body (represented by the length of the line drawn through the neural canal) and of the cross-sectional shape of that section through the glycogen-body level of the spinal cord in which the area of the glycogen body is of maximum size (see text). All the cross-sections were projected at the same magnification upon the ground glass of the tracing table, were outlined on tracing paper, and were subsequently transferred to this figure. Wherever possible, tracings of two or more specimens were superimposed to indicate the variation encountered. Sections of control embryos are to the right, sections of embryos submitted to unilateral leg-bud extirpation are in the middle, sections of embryos submitted to bilateral leg-bud extirpations are to the left.

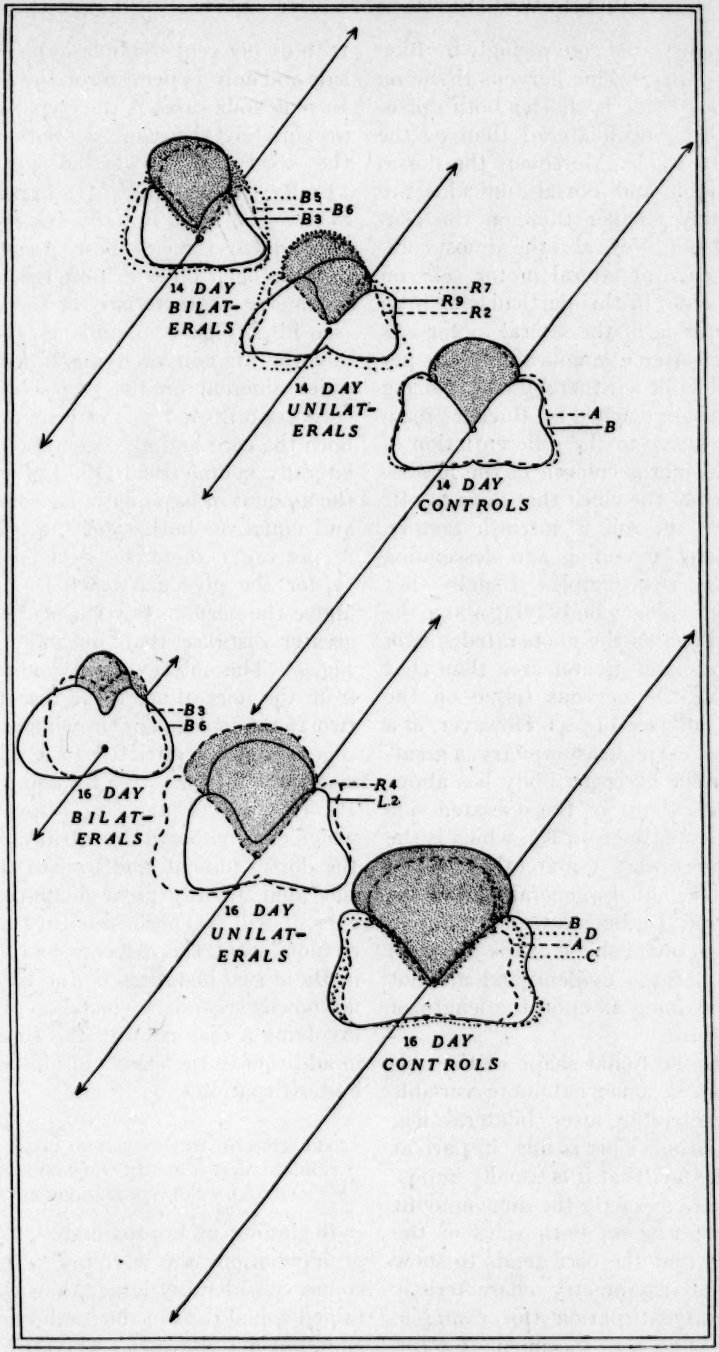
B5
B6
B3
14 DAY BILAT-ERALS
R7
R9
R2
14 DAY UNILAT-ERALS
A
B
14 DAY CONTROLS
B3
B6
16 DAY BILAT-ERALS
R4
L2
16 DAY UNILAT-ERALS
B
D
A
C
16 DAY CONTROLS

FIG. 4.—PART II

been demonstrated convincingly by Bueker (1943, 1947). The nervous tissue on the operated side is shorter both dorsoventrad and mediolaterad than on the nonoperated side. Moreover, the dorsal gray column and dorsal funiculus are considerably smaller than on the contralateral side. Note also the almost complete absence of lateral motor cells on the right side; in this particular embryo the hypoplasia of the lateral motor column was extreme, amounting to 94 per cent (see Table 1), thereby emphasizing the conclusion reached by Bueker (1943, p. 110) relative to the differentiation of the lateral motor column in the lumbosacral cord of the chick that "our results imply that the role of intrinsic factors, that is, long ascending and descending fiber tracts, is negligible." Usually that part of the glycogen body lying above the nervous tissue on the unoperated side of the embryo is of greater area than that part above the nervous tissue on the operated side (see Fig. 4). However, in a few cases of extreme asymmetry, a greater part of the glycogen body lies above the nervous tissue of the operated side (see Fig. 4, specimen 14 R9, which is the one of the 14-day unilaterals outlined with a solid line). In general, the effects of unilateral leg-bud extirpation upon the cross-sectional shape of the glycogen body are not too evident and are not nearly so striking as upon the length of this structure.

The cross-sectional shape of the glycogen body is somewhat more variable and unpredictable after bilateral leg-bud extirpation. This is due, in part at least, to the fact that it is usually impossible to remove exactly the same amount of the periphery on both sides of the body, such that the cord tends to show some of the asymmetry characteristic of unilateral extirpation (for example, see Pl. I, Fig. 13, specimen 16.0 B6, with 91 per cent hypoplasia on the right side and only 34 per cent on the left side). In only four cases is the hypoplasia approximately the same on both sides of the cord: 10.5 B (80–86 per cent); 14.0 B3 (89–89 per cent); 14.0 B5 (80–85 per cent); 16.0 B3 (100–100 per cent). In the latter three of these cases the cord and glycogen body exhibit the bilateral symmetry characteristic of the controls (see Pl. I, Figs. 8, 9, and 11). Occasionally the amount of hypoplasia may be quite different on the two sides of the cord (14.0 B6: 90–75 per cent), and yet both the cord and glycogen body are bilaterally symmetrical (Pl. I, Fig. 7). If the amount of hypoplasia is considerable and equal on both sides (14.0 B3: 89–89 per cent), there is a decided tendency for the glycogen body to protrude above the nervous portion of the cord a greater distance than normally (Pl. I, Fig. 9). The only extreme modifications from the normal are to be found in the two 16-day specimens, in which the glycogen body is restricted to a relatively small cap of cells (16.0 B3 and 16.0 B6, Pl. I, Figs. 11 and 13, respectively), which does not extend ventrally between the dorsal funiculi and toward the neural canal for any great distance. However, it will be emphasized in the "Discussion" that the extreme modification in these two instances is due to certain unknown secondary factors, probably involving a distortion of the roof plate, in addition to the effects of bilateral leg-bud extirpation.

V. RELATION BETWEEN SIZE AND SHAPE OF VERTEBRAL CANAL AND SIZE AND SHAPE OF SPINAL CORD AT GLYCOGEN-BODY LEVEL

Beginning at approximately 8½ days of incubation, the vertebral canal becomes considerably larger than the contained spinal cord in the lumbosacral region, but not elsewhere. The ratio of the

volume of the spinal cord at the glycogen-body level to the volume of the adjacent vertebral canal has been calculated by Watterson (1949) for various developmental stages of normal embryos; these values are recorded in his Table IV and are plotted on his Figure 5 and on our Figure 5 (*solid line*). It will be noted that from $8\frac{1}{2}$ to 10 days the cord occupies approximately six-tenths of the vertebral canal; from $10\frac{1}{2}$ to 13 days it occupies approximately seven-tenths of the canal; thereafter the vertebral canal enlarges more rapidly than the spinal cord, such that the ratio of the latter to the former drops to approximately five-tenths. The same ratio has been calculated for the operated embryos; these values are recorded in Table 4 and are plotted on Figure 5; as before, the values for the unilaterals (*broken line*) are plotted separately from those for the bilaterals (*dotted line*). It will be noted that the three curves fall very close together; special attention should be called to the proximity of the points on the sixteenth day of incubation, since this is the only stage at which the volumes of the glycogen bodies and adjacent nervous tissue are markedly affected by the operations.

Although the ratios of the volume of the spinal cord to the volume of the adjacent vertebral canal have not been markedly affected by the operation, the *shape* of the vertebral canal has been considerably affected in some instances. Even in normal embryos the cross-sectional shape of the vertebral canal is quite variable (Watterson, 1949, Pls. III and IV). However, in the operated embryos the departure from the normal variation in shape may be considerable. An extreme departure is seen in Plate I, Figure 9. Here is a case in which the nervous portion of the spinal cord is considerably shortened from right to left, and the glycogen body is considerably deepened dorsoventrally. Note the narrowness of the vertebral canal, relative to its width, in comparison with other specimens illustrated in Plate I, and note the straight left wall of the vertebral canal, associated with the relatively straight left side of the spinal cord. No such shape of the vertebral canal has ever been encountered in control embryos. Or examine Plate I, Figure 10, where two glycogen bodies (see "Discussion") are present; note how space has been made available for this extra glycogen body by widening of the vertebral canal to the left and dorsad. Somewhat more posteriorly in the same specimen (Pl. I, Fig. 11), just one section caudad to the most posterior one through the accessory glycogen body, the curvature of the left wall of the vertebral canal becomes more rounded, more "normal" in appearance. Again, in Plate I, Figure 13, where the larger part of the glycogen body lies

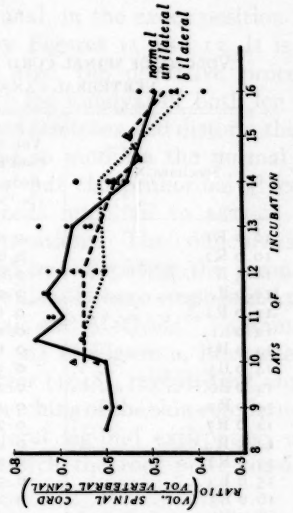

FIG. 5.—Comparison of the ratio of the volume of the glycogen-body level of the spinal cord to the volume of the adjacent vertebral canal in normal and operated embryos.

above the left side of the nervous tissue, note that the expansion of the dorsal part of the vertebral canal is to the left. The conclusion reached by Watterson (1949) with respect to the shape of the vertebral canal in normal embryos seems to hold quite well for these operated embryos, viz., that "the general shape of the vertebral canal corresponds quite precisely to the outlines of the glycogen body and nervous portion of the cord." However, there are occasional exceptions: in Figure 12 the correspondence between the shape of the cord and that of the vertebral canal is evident on the right side; but on the left there seems to be no such agreement. It should be noted, however, that the dorsal part of the left side of the cord is very abnormal; it may be that some resorption of the dorsal part of the cord has occurred here at an earlier stage and that the agreement between the shape of the spinal cord and the shape of the vertebral canal was greater at an earlier stage than at 16 days, when this specimen was sacrificed. More posteriorly in the same specimen (Fig. 13) the agreement between the shape of the vertebral canal and that of the spinal cord is pronounced.

TABLE 4

VOLUME OF SPINAL CORD AT GLYCOGEN-BODY LEVEL AND OF ADJACENT VERTEBRAL CANAL AND RATIO OF FORMER TO LATTER*

Specimen No.	Vol. Spinal Cord at Gly. Body Level ($In.^3 \times 10^{-3}$)	Vol. Adj. Vertebral Canal ($In.^3 \times 10^{-3}$)	Ratio Vol. Spinal Cord / Vol. Adj. Vert. Can.	Av. Ratio
10.0 R1	0.0805	0.130	0.62	0.645
10.0 R2	0.0637	0.095	0.67	
10.5 B	0.0373	0.057	0.65	0.65
12.0 B	0.095	0.159	0.60	0.60
12.0 R1	0.117	0.189	0.62	0.645
12.0 L1	0.146	0.218	0.67	
14.0 B3	0.141	0.241	0.59	0.61
14.0 B5	0.354	0.537	0.66	
14.0 B6	0.207	0.354	0.58	
14.0 R2	0.203	0.354	0.57	0.575
14.0 R7	0.278	0.482	0.58	
14.5 R	0.283	0.548	0.52	0.52
15.0 R2	0.262	0.477	0.55	0.545
15.0 R3	0.529	0.978	0.54	
16.0 B3	0.096	0.251	0.38	0.415
16.0 B6	0.121	0.267	0.45	
16.0 R4	0.434	0.983	0.44	0.465
16.0 L2	0.401	0.815	0.49	

* See note to Table 2.

DISCUSSION

Examination of Figure 1, *A*, reveals that the growth curves of the glycogen bodies in normal and operated embryos are very similar until the fifteenth day of incubation. Thereafter, the curve for the embryos submitted to unilateral leg-bud extirpation levels off; by contrast, the curve for the embryos submitted to bilateral leg-bud extirpation drops considerably. Actually, this latter curve is misleading; it does not necessarily imply that the glycogen bodies of the embryos which underwent bilateral leg-bud extirpation reached a maximum size at 14–15 days of incubation and then regressed; if we had been able to measure the vol-

ume of the glycogen bodies of these two specimens at intervals during their development, it is quite likely that these values would have been consistently small throughout. Thus these two specimens do not fall into line with the others examined, and it seems quite evident that other secondary factors, in addition to the effects of reduction of the periphery, must have exerted an influence on these two glycogen bodies.

I. MODIFICATIONS OF GLYCOGEN BODY WHICH CANNOT BE ATTRIBUTED TO REDUCTION OF PERIPHERY ALONE

Sections through the glycogen-body level of the two 16-day specimens which had undergone bilateral leg-bud extirpation prior to 65 hours of incubation are illustrated in Figures 11 and 13. The glycogen bodies belonging to these operated embryos are extremely narrow from right to left and are extremely short dorsoventrally; they protrude above the dorsal funiculi a shorter distance than normally. Moreover, they are extremely short anteroposteriorly (Fig. 3). But perhaps *their most unusual characteristic is their failure to extend ventrad between the dorsal funiculi to reach the neural canal.* Associated with this lack of ventral extension of the glycogen body is the approximation of the dorsal gray columns, a relationship characteristic of other levels of the cord but not of the normal lumbosacral cord. To account for the foregoing relationships, it is necessary to examine the initial stages in the formation of the glycogen body in normal embryos.

At the time when glycogen-storing cells are first recognizable within the roof plate of the glycogen-body level of the neural tube of normal embryos (at approximately 8 days of incubation), they are most concentrated dorsally, just beneath the pia mater (see Watterson, 1949, Fig. 1*B*), and they gradually diminish in number and size as they approach the neural canal on each side of the mid-line. If, for some reason, the glycogen-storing cells failed to extend ventrad toward the neural canal at this time and remained restricted to the region just beneath the pia mater, one might anticipate that the resulting glycogen body would be restricted to a cap of cells some distance removed from the neural canal, in the exact position illustrated by Figures 11 and 13. It is conceivable that the operative procedure necessary for removal of both leg buds sometimes stretches and distorts the roof plate and so modifies the normal relationships that the primordial glycogen-storing cells may fail to assume their proper positions. The difficulties encountered in extirpating the second leg bud have already been emphasized under "Materials and Methods." Examination of stage 3n4 of Figure 2, illustrated by Hamburger (1948), reveals that any unusual stretching of the skin ectoderm during bilateral leg-bud extirpation might easily stretch the roof plate considerably.

That the roof plate has probably been injured in some way in these two specimens is evident if embryo 16.0 B6 (Fig. 13) is studied in detail. Actually, although every effort was made to remove both leg buds completely, the attempt in this instance was not successful, since many lateral motor cells are to be seen on the left side of the cord; if only the effects of reduction of the periphery had modified this glycogen body, the latter should resemble the glycogen body of embryos submitted to unilateral leg-bud extirpation (Fig. 6). In embryo 16.0 B6 the glycogen body first appears on slide 55, extends through 33 sections in the asymmetrical position illustrated in Figure 12, then disappears completely, only

to reappear 6 sections later in the relatively symmetrical position indicated in Figure 13. More posteriorly still, a narrow tongue of glycogen-storing cells does extend ventrad until it reaches the neural canal, but only for the distance of 4 sections; then the sections again resemble Figure 13, but more posteriorly the glycogen body again dips toward the neural canal for the distance of 3 sections; again the sections resemble Figure 13. Finally, in the last few sections through this abnormal glycogen body the glycogen-storing cells extend all the way to the neural canal. It should be emphasized, however, that, although the distortion was sufficient to prevent the formation of a normal glycogen body, it was not sufficient to prevent formation of complete vertebrae; this would suggest that the injury of the roof plate was not a direct one but one exerted upon it from a distance.

Specimen 16.0 B_3 is very similar to 16.0 B6; in the former, however, the glycogen body never extends ventrad to reach the neural canal. It also differs from embryo 16.0 B6 in that an accessory glycogen body has formed (to the left in Fig. 10). The two glycogen bodies lie side by side over a distance of 20 sections; and if they are connected at all, it is by a narrow bridge of glycogen-storing cells in a single section. Posterior to the accessory glycogen body there is only the single mid-line glycogen body illustrated in Figure 11. The source of the accessory glycogen body is obscure; whether some primordial glycogen-storing cells have been displaced to the left from their usual mid-line position (owing to some distortion of the roof plate exerted by the operative procedure) or whether glycogen-storing cells have, for some reason, originated at a point where they are not formed under normal circumstances cannot be decided on the basis of this one specimen alone. Again the distortion of the roof plate was not sufficient to prevent the formation of complete vertebrae.

II. MODIFICATIONS OF GLYCOGEN BODY WHICH MAY BE ATTRIBUTED TO REDUCTION OF PERIPHERY

What, then, are the effects of unilateral and bilateral leg-bud extirpation upon the development of the glycogen body of the chick spinal cord?

1. There is a definite reduction in length of the glycogen body compared with the normals (see Fig. 3). This may mean that the anteroposterior extent of the roof plate which becomes populated with future glycogen-storing cells is less in operated than in normal embryos; or it may be that the anteroposterior extent of the primordium of the glycogen body is the same in both instances but that, under the conditions of reduction in size of certain components of the cord resulting from reduction of the periphery, the most anterior and most posterior parts of this primordium fail to differentiate.

2. The glycogen body lacks bilateral symmetry after unilateral leg-bud extirpation and may be asymmetrical after bilateral leg-bud extirpation if the removal of the leg bud is less complete on one side than on the other. However, if removal of the leg buds is equally complete on both sides, the glycogen body is bilaterally symmetrical.

3. There is perhaps some tendency for the dorsal funiculi to be less widely separated from one another after bilateral leg-bud extirpation (see especially Fig. 9); this would offer some slight support for the ideas of Imhof (1905) and Kappers (1924) that the dorsal columns at this level of the cord become separated from one another owing to some traction exerted by the roots of the sciatic nerve. Presumably as a result of the reduction in the size of the dorsal roots, which is due, in turn, to a reduction in the size of

the periphery, any such traction might be lessened. However, Figure 8 illustrates another case of bilateral leg-bud extirpation, rather complete on both sides (80–85 per cent hypoplasia), which does not result in any approximation of the dorsal columns. Whether in normal development the expanding glycogen body *forces* the dorsal columns apart or whether they separate from one another because of the growth characteristics within the nervous portion of the cord or whether traction of the dorsal roots of the sciatic nerve is the major activity in separating the dorsal columns has not yet been demonstrated. However, specimens such as those illustrated in Figures 11 and 13 would seem to suggest that expansion of the glycogen body to assume its normal size is an important factor in forcing the dorsal columns apart. However, it should be noted that in these two instances the nervous portion of the cord is rather disorganized also, suggesting that the growth characteristics within the nervous portion of the cord have also been interfered with. Surgical removal of the primordium of the glycogen body should give a clue as to which of the above factors is the major one responsible for the peculiar morphology of the lumbosacral spinal cord of birds.

4. There is little, if any, reduction in volume of the glycogen body prior to 15 days of incubation. The leveling-off at 15 days of the curve of growth of the glycogen bodies of specimens submitted to unilateral extirpation without any evidence of distortion of the roof plate may mean that the effects of reduction of the periphery upon the size of the glycogen body are exerted only during the later days of incubation. It will be necessary to examine other operated embryos which have not been sacrificed until about the time of hatching. If it can be demonstrated that beyond the fifteenth day the volume of the glycogen body is consistently less after both unilateral and bilateral leg-bud extirpations, we shall have an interesting correlation with a change in the nature of the growth of the glycogen body which occurs at about the fifteenth day of incubation. Watterson (1949), states:

> The period between 8 and 15 days of incubation is largely concerned with growth due to increase in the number of cells within the glycogen body, rather than increase in size of the individual cells. Thereafter the proliferation drops off steadily, whereas the individual cells increase in size rapidly, such that the increase in volume of the glycogen body after 15 days of incubation is due almost entirely to growth of individual cells.

It appears, then, that the reduction in the size of certain components of the spinal cord and sciatic nerve which can be accomplished by leg-bud extirpation has little effect upon that part of the growth of the glycogen body due largely to cell proliferation; there is an indication that the above reduction may exert a considerable effect upon the later stages of growth of the glycogen body, owing largely to increase in the size of individual cells.

5. When this study was initiated, it was thought that the reduction of the periphery and the resulting reduction of certain parts of the spinal cord would considerably reduce the total volume of nervous tissue adjacent to the glycogen body, and that a comparison of the ratio

$$\frac{\text{Vol. glycogen body}}{\text{Vol. adj. nerv. tiss.}}$$

in operated and control embryos would reveal whether the volume of the glycogen body is controlled in any specific way by the volume of the adjacent nervous tissue or whether the two components of the spinal cord develop relatively independently of each other. However, the total volume of the nervous tissue

adjacent to the glycogen body was scarcely reduced, if at all, prior to 16 days of incubation, in spite of the reduction in length of the glycogen body (see Fig. 1, *B*). Consequently, the similarity of the ratio

$$\frac{\text{Vol. glycogen body}}{\text{Vol. adj. nerv. tiss.}}$$

at various developmental stages in operated and control embryos prior to the sixteenth day tells us nothing except to verify these ratios as originally calculated by Watterson (1949) for normal embryos. Moreover, as emphasized in the first part of the "Discussion," the very low ratio obtained in the two 16-day embryos submitted earlier to bilateral leg-bud extirpation can have no significance, since, in all probability, the roof plate was injured or distorted in some way by the operative procedure, upsetting any normal relationship that might have existed between volume of the glycogen body and volume of the adjacent nervous tissue. However, in the two 16-day embryos submitted earlier to unilateral leg-bud extirpation, the ratio has dropped somewhat compared with the controls (Fig. 2), and this may possibly suggest that at least the second phase of growth of the glycogen body—that due to increase in size of individual cells—is relatively independent of the volume of the adjacent nervous tissue. Only experiments in which the operated embryos survive to stages later than 16 days will demonstrate whether such independence actually exists or whether the independence suggested here is due to sampling error.

III. FACTORS AFFECTING THE SIZE AND SHAPE OF THE VERTEBRAL CANAL

There are on record several studies of abnormal vertebral columns and skulls in man, which demonstrate that the size of the vertebral canal and cranial cavity is controlled in some way by the size of the contained spinal cord and brain. For example, Herren and Edwards (1940) described certain cases of duplication of the spinal cord (diplomyelia) and stated (p. 1205) that "the spinal [vertebral] canal may show an abnormal appearance, varying from a moderate degree of dilatation at the level of the duplication of the cord to partial doubling." They concluded that the diplomyelia represents a primary alteration of the spinal cord and not a secondary condition due to the abnormality of the spine, since the spinal cord develops long before the vertebral column during embryonic life. Walker (1944) described several cases of dilatation of the vertebral canal associated with congenital anomalies of the spinal cord and emphasized that, although it is well known that the spinal (vertebral) canal may be markedly dilated as the result of an intraspinal neoplasm, it is not generally recognized that an equally pronounced dilatation may occur at any level as the result of a congenital anomaly of the cord structures. Troitzky (1932) and Weidenreich (1941) both emphasized the primary importance of the size of the brain in controlling the size of the cranial cavity; and the attempts of Washburn (1947) to affect the form of the brain case by removal of temporal and nuchal muscles of newborn rats led him to conclude (p. 247): "The internal form of the brain case was not altered by the removal of the temporal muscle alone and was affected in only the most minor way by the removal of both temporal and nuchal muscles." Feller and Sternberg (1934) stated (p. 610) that ". . . die Ausbildung der Wirbelbogen und der Schädelkapselknochen vom Rückenmark, bzw. vom Gehirn abhängig ist"; and Williams (1942) believed that his experiments on the development of the cervical verte-

brae of the chick supported the conclusion reached by Feller and Sternberg (p. 633): "dass die Wirbelsäule aus zwei entwicklungsmechanisch verschiedenen Anteilen besteht, einerseits aus den von der Ausbildung des Zentralnervensystems beeinflussten Wirbelbogen, andererseits aus der in ihrer Entwicklung von der Chorda dorsalis abhängigen Wirbelkörperreihe."

However, even though it is accepted in principle that the size of the spinal cord in some way determines the size of the vertebral canal, the *mechanism* by which this control is exercised is unknown. Mechanical pressure of the abnormally enlarged spinal cord against the rudiments of the neural arches does not appear to be a factor in the dilatation of the vertebral canal in man, since Walker (1944) observed in his study of congenital anomalies of the spinal cord that "there was no evidence of spinal cord compression either by lumbar puncture or at the time of operation. In fact, in the first two cases of myelodysplasia there was much more room in the subarachnoid space than usual" (p. 581). Similarly, at the glycogen-body level of the chick vertebral column in both the normal and the experimental embryos, not only is the size of the vertebral canal greater than elsewhere by an amount necessary to accommodate the increased size of this level of the cord, but it is excessively greater, so much so that the spinal cord does not begin to occupy all the space available within the vertebral column. Any influence of the size of the cord upon the size of the vertebral canal must be exerted over some distance under these circumstances. And yet a very precise relationship apparently exists between the volume of the glycogen-body level of the cord and the volume of the adjacent vertebral canal, as can be seen from Figure 5, since the ratios of the former to the latter are very similar in operated and normal embryos. Although this is not very surprising in stages prior to 16 days of incubation (owing to the fact that, prior to this stage, leg-bud extirpation has little effect upon the total volume of the glycogen body and adjacent nervous tissue), it is very striking in the two 16-day specimens which had undergone bilateral leg-bud extirpation; in spite of the resulting extreme reduction in the volume of the glycogen body and adjacent nervous tissue, the ratio of the volume of the spinal cord to that of the adjacent vertebral canal remained quite close to normal. At other levels of the chick vertebral column the spinal cord more nearly fills the vertebral canal. It would be interesting to transplant lumbosacral levels of the spinal cord to higher levels of the body in order to determine whether the primordia of the neural arches at those levels responds equally well to the size-controlling activities of the lumbosacral cord.

SUMMARY

1. This study represents a preliminary attempt to analyze the factors responsible for the development of the glycogen body of the chick spinal cord. The size of certain components of the spinal cord and sciatic nerve has been reduced indirectly by radical leg-bud extirpation in an attempt to determine whether the size of these components controls the development of the glycogen body in any way.

2. The extreme hypoplasias of the lateral motor column obtained by Bueker (1943, 1947) after unilateral leg-bud extirpation have been verified. In cases of bilateral leg-bud extirpation the lateral motor columns may be completely lacking on both sides.

3. There is a definite reduction in length of the glycogen body following

both unilateral and bilateral leg-bud extirpation.

4. The glycogen body is asymmetrical after unilateral leg-bud extirpation and may be asymmetrical after bilateral leg-bud extirpation if the removal of the leg bud is less complete on one side than on the other. The glycogen body in normal embryos and in embryos with equal reduction of the periphery on both sides is bilaterally symmetrical.

5. There is little, if any, reduction in volume of the glycogen body prior to 15 days of incubation; but there is a suggestion that after the fifteenth day the volume of the glycogen body will be smaller in operated, than in normal, embryos. This is perhaps correlated with a change in the growth characteristics of the glycogen body at about the fifteenth day, as demonstrated by Watterson (1949). It will be necessary to examine the effects of leg-bud extirpation upon older embryos before this relationship can be clarified.

6. The only extreme modifications of the glycogen body occurred in two 16-day embryos which had undergone bilateral leg-bud extirpation prior to 65 hours of incubation. However, detailed study of these specimens suggests that at the time of the operation the roof plate was accidentally modified by stretching and distortion, so that the primordial glycogen-storing cells failed to assume their proper positions. The resulting abnormalities of the glycogen bodies are, therefore, only in part due to the reduction of the periphery.

7. Since reduction of the periphery exerts so little effect upon the development of the glycogen body, the causal factors which control the development of the glycogen body, if they reside outside the neural tube, must lie close to the latter, in either the somites, the notochord, or the neural crest.

8. The size of the glycogen-body level of the cord appears to control precisely the size of the adjacent vertebral canal. However, the control of the former over the latter cannot be due to mechanical pressure alone, since the size of the vertebral canal greatly exceeds that of the contained spinal cord in the lumbosacral region. Any influence of the size of the cord upon the size of the vertebral canal must be exerted over some distance under these circumstances.

9. The shape of the vertebral canal is determined to a considerable extent by the shape of the spinal cord at the glycogen-body level, again by factors acting over some distance.

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PLATE I

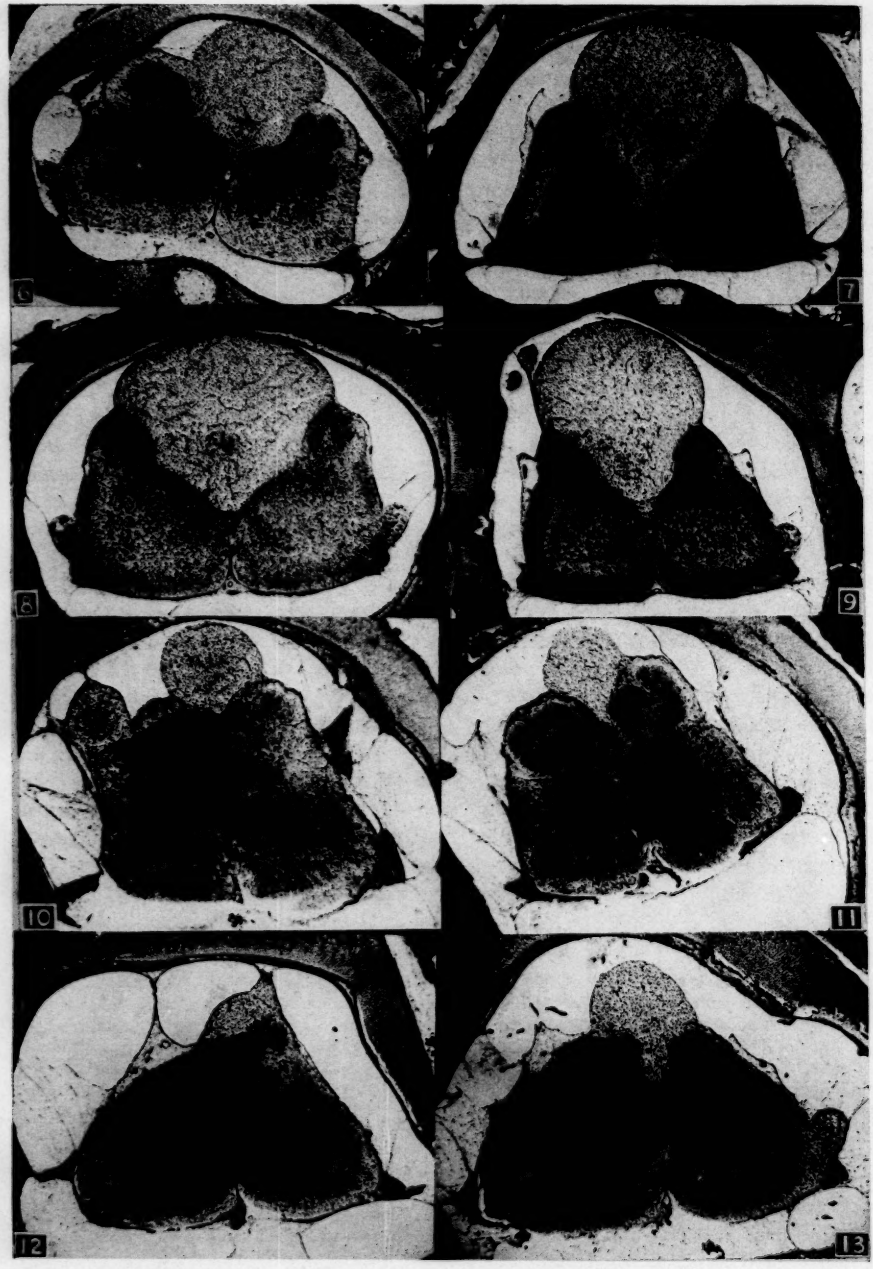

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PLATE I

FIGS. 6–13 × *ca.* 23.

FIG. 6.—Specimen 12.0 R1.

FIG. 7.—Specimen 14.0 B6.

FIG. 8.—Specimen 14.0 B5.

FIG. 9.—Specimen 14.0 B3.

FIG. 10.—Specimen 16.0 B3. Note the accessory glycogen body to the left (see text).

FIG. 11.—Specimen 16.0 B3. More posterior section (see text).

FIG. 12.—Specimen 16.0 B6 (see text).

FIG. 13.—Specimen 16.0 B6. More posterior section (see text).

THE AMINO ACID REQUIREMENTS OF TETRAHYMENA GELEII (E)[1]

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SOME years ago attempts were made to determine the specific amino acid demands of a particular strain of *Tetrahymena geleii* (E) without success (Elliott, 1935*b*; Hall and Elliott, 1935). One of several possible reasons for failure was that one of the essential amino acids (methionine) was unavailable and another (threonine) was unknown. Undoubtedly, other nutritional needs, such as certain growth factors, prohibited success at this early period. In recent years the precise amino acid needs of another strain of this ciliate, strain W, have been determined by Kidder and his co-workers (Kidder and Dewey, 1945*a*, *b*, 1947). It seems important that more exact information be gathered concerning the nutritional requirements of other strains of this protozoan, which has become an important experimental animal in recent years.

This investigation was undertaken, therefore, to determine the amino acid requirements of strain E and the concentration of the several essential amino acids that support optimal growth.

MATERIALS AND METHODS

The organism used in this investigation was isolated in 1932 (Elliott, 1933) and has since been employed in several research projects. It was formerly known

[1] This investigation was supported in part by a grant from the Horace H. Rackham School of Graduate Studies.

as *Colpidium striatum* but has more recently been shown to be a strain of *T. geleii* (Furgason, 1940); the designation of strain E was assigned by Kidder and Dewey (1945*b*). It is cultured free from all other microörganisms in 15×125-mm. Pyrex test tubes. All estimates of growth were carried out turbidimetrically with the use of a Klett-Sommerson photoelectric colorimeter which has been adapted to this type of work (Elliott, 1949). Readings were taken in Klett units from the inoculated tubes at the beginning of the experiment and again at the end, or at regular intervals throughout the course of the experiment. The difference between these readings was taken as indicative of the amount of growth. Klett units[2] are whole numbers that are proportional to optical density, the latter being a function of the number of suspended cells. The Klett units were used directly rather than translated into optical densities, which are fractional units. No effort has been made to interpret these units in numbers of organisms because relative information is all that is needed in this investigation.

TABLE 1

Substance	Micrograms per Milliliter
Dextrose	1,000
Sodium acetate	1,000
$MgSO_4 \cdot 7H_2O$	100
K_2HPO_4	100
$CaCl_2 \cdot 2H_2O$	50
$FeCl_3 \cdot 6H_2O$	1
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	25
$CuCl_2 \cdot 2H_2O$	5
$MnCl_2 \cdot 4H_2O$	0.05
$ZnCl_2$	0.05
Ca pantothenate	0.10
Nicotinamide	0.10
Pyridoxine HCl	2.00
Riboflavin	0.10
Pteroyltriglutamic acid	0.01
Biotin	0.0005
Thiamine HCl	1.0
Choline Cl	1.0
Yeast nucleic acid	100
Protogen	1.0 unit

The composition of the basic culture medium used in all these experiments, exclusive of the amino acids, is shown in Table 1. This basic medium is the same as that reported by Kidder and Dewey (1949), with the following exceptions. Instead of pteroylglutamic acid, pteroyltriglutamic was used because of its higher molar activity. The concentration of unity for $FeCl_3$ instead of 1.25 γ per milliliter seemed adequate. Tween 85 had so little stimulatory effect that it seemed inadvisable to use it because of its troublesome opalescence, which interferes with readings in the colorimeter. Protogen was used in the concentration of one unit per milliliter, as recommended in a private communication by Dr. E. L. R. Stokstad, of the Lederle Laboratories. Not all the substances listed in the basic medium are essential for growth of strain E, but, since the precise needs are being investigated now, it was thought advisable to include all those that produce optimal growth of strain W.

The Factor II used in the original experiments was prepared according to the method reported by Kidder and Dewey (1947). The initial substances from which it was extracted were Cerophyll and Liver Fraction L; the former substance seemed to contain fewer amino acids and hence was used for all the preliminary work. It was found that, no matter what the source of Factor II, amino acids were always present in small quantities, as indicated by growth in tubes without the individual essential amino acids. The

[2] The relationship of Klett units to optical density is indicated by the formula

$$R = \frac{1000 \times D}{2},$$

where R is the Klett unit and D the optical density.

more recent experiments reported here were done with protogen instead of Factor II, and the results have been more precise and indicate that this factor contains no amino acids.

The basic medium was prepared in large quantities, to which amino acids were added in graded amounts, depending on the experiment. The various concentrations were made in triplicate tubes. With the exception of the essential amino acid series, inoculations were made with pipettes; a 0.2-ml. inoculum from a heavy suspension of washed ciliates in their logarithmic growth phase maintained in purified medium was used. The organisms were washed several times with sterile distilled water before serving as the inoculum; this procedure allowed a carry-over of very little, if any, of the original medium. Washing did not injure the Protozoa in any way that could be detected with the microscope or in their subsequent growth. By using relatively high concentrations of organisms at the beginning of the experiments, the results could be read much sooner and were more consistent. It was difficult to obtain uniform initial inoculations with a loop. The results of the first two tubes cultured simultaneously were checked against each other; the third tube was a loop transfer which was used only to confirm the results of the first two tubes. All experiments were repeated several times, and the most consistent series were selected for reporting here.

All media were prepared in double-distilled water, and the pH was set at 7.4 at the start of the experiments. A test series demonstrated that growth is good over a pH range of 6.5–7.5 but that optimal growth in purified media occurs either at 6.5 or at 7.5; hence 7.4 was arbitrarily selected. All cultures were incubated in the dark at a constant temperature of 25° C.

The amino acids were prepared in 10 mg/ml solutions in double-distilled water; the proper dilutions were then prepared, with the basic medium as the diluent. Rather than starting the amino acid levels based on the analysis of gelatin or casein, arbitrary concentrations were selected, and within each series these were the same for all the amino acids except the one for which the dose response curve was determined. The levels selected were 100, 400, and 800 γ/ml, each level constituting a series which is designated by the concentration number, e.g., 100, 400, and 800 series.

The amino acids and growth factors, with the exception of protogen, were supplied by Merck and Company. The protogen was obtained from Dr. E. L. R. Stokstad. The Factor II preparations were extracted from Cerophyll, supplied by the Cerophyll Laboratories, and from Liver Fraction L, furnished by the Wilson Laboratories. The author wishes to express his gratitude to these various companies and to Dr. Stokstad for their generous support.

EXPERIMENTAL

Essential amino acids.—Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are essential for growth of strain W; while serine was routinely employed for optimal growth, it is stimulatory only (Kidder and Dewey, 1945). Early experiments without serine indicated that strain E could not synthesize this amino acid. Hence this series was run with this amino acid together with the ten essential for the rat and strain W, omitting them one at a time. In the first series of experiments a Factor II preparation was employed instead of protogen. Loop transfers were used in this series rather than the heavy pipette inocula which were employed in

all subsequent experiments. From Figure 1 it is obvious that growth has occurred even after the third transfer in tubes without lysine, phenylalanine, threonine, tryptophan, and valine. This indicates that either the ciliate can synthesize these amino acids at a slow rate or that the amino acids were being added with the Factor II or as contaminants of the other amino acids. When protogen became available, these experiments were repeated, and no growth occurred after the second transfer in any of the tubes in which any one of the eleven amino acids was omitted. It would appear, then, that the ten amino acids essential in the nutrition of the rat and serine are essential for this strain of *T. geleii*.

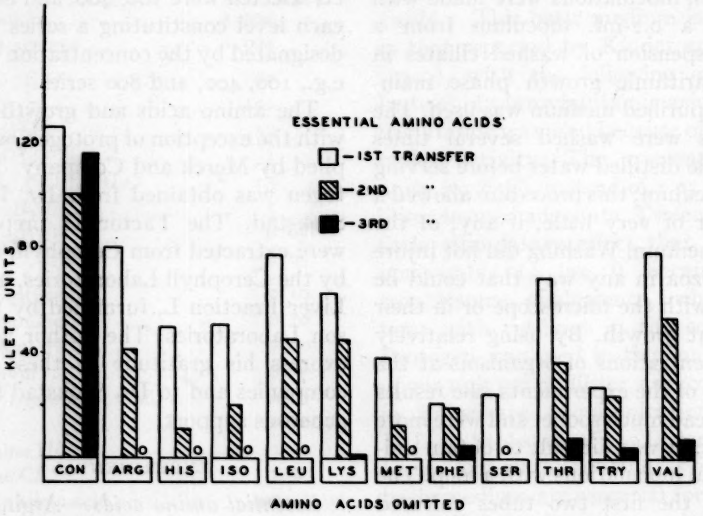

FIG. 1.—Growth is recorded in Klett units for three transfers in which the individual amino acids are omitted singly.

CONCENTRATION SERIES

The 100 series.—In this series of experiments to determine the effective concentration of the essential amino acids that support the best growth, the basic level of amino acids was arbitrarily set at 100 γ/ml. The basic culture medium with protogen was used in this series as well as in all subsequent experiments. Dose response curves were run on each of the amino acids through five different concentrations (10, 25, 50, 75, 100). The first set of tubes contained none of the amino acids being investigated. It will be noted (Table 2) that some growth did occur in all the tubes in which there was none of the amino acid under investigation; this is probably due to slight reserves in the cells, because subsequent loop transfers into the third set of tubes showed no growth whatever in any of them. There is no evidence that serine is stimulatory only, in so far as these experiments indicate.

The 400 series.—In this series the basic level of amino acids was held at 400 γ/ml, and each of the eleven amino acids varied in concentrations through five steps (10, 50, 100, 200, 400). Slight growth is again observed here (Table 3) in the control tubes where the amino acid

under investigation is omitted; furthermore, no stimulation is observed with serine, although lysine and tryptophan showed good growth at low concentrations.

The 800 series.—In this series the basic level of amino acids was set at 800 γ/ml, and the eleven amino acids varied in concentrations through five steps (20, 100, 200, 400, 800). Here again (Table 4), as in the two preceding series, growth occurred to a slight degree in the control tubes. Since there was no concentration lower than 20 γ/ml, growth with all the amino acids was very much the same at this level. The lowest concentration was too high to show any stimulatory effects if they were present.

TABLE 2*

THE 100 SERIES

Amino Acids	Concentration of Amino Acids in Micrograms per Milliliter (Klett Units)					
	0	10	25	50	75	100
l-Arginine	10	15	41	56	55	**62**
l-Histidine	15	51	57	58	**62**	50
dl-Isoleucine	16	70	**82**	80	74	72
l-Leucine	6	63	**75**	61	58	55
l-Lysine	20	55	**65**	58	52	48
dl-Methionine	8	45	56	**66**	52	40
dl-Phenylalanine	10	45	**68**	63	50	40
dl-Serine	7	32	54	55	**62**	50
dl-Threonine	10	31	43	**60**	56	45
l-Tryptophan	10	36	58	**70**	59	51
dl-Valine	7	43	54	**72**	54	47

* Growth is indicated in Klett readings at the various concentration levels. Optima are shown in boldface type.

TABLE 3*

THE 400 SERIES

Amino Acids	Concentration of Amino Acids in Micrograms per Milliliter (Klett Units)					
	0	10	50	100	200	400
l-Arginine	0	5	36	60	61	**68**
l-Histidine	10	35	70	**75**	72	68
dl-Isoleucine	0	24	**68**	60	62	60
l-Leucine	5	30	**72**	67	67	62
l-Lysine	10	62	74	**80**	75	70
dl-Methionine	0	25	73	**78**	72	70
dl-Phenylalanine	10	30	58	**66**	62	60
dl-Serine	0	3	64	75	**78**	63
dl-Threonine	0	7	45	64	**81**	78
l-Tryptophan	7	70	72	**78**	72	68
dl-Valine	0	33	60	**75**	73	58

* Growth is indicated in Klett readings at the various concentration levels. Optima are shown in boldface type.

Comparative series.—The concentrations of the various amino acids that supported optimal growth in the three preceding series are assembled in Table 5. Since the total amino acid level of the 800 series was about the same as a 0.5 per

cent casein solution, which has been shown to support adequate growth, it was decided to raise the 100 and 400 series to that level by multiplying all the concentrations by 4 and 1½, respectively. This made the total amino acid levels studied for comparison. The missing amino acids in gelatin (methionine, tryptophan, and valine) were added in the same concentrations as in the 800 series.

The amino acid levels were set at these

TABLE 4*

THE 800 SERIES

Amino Acids	Concentration of Amino Acids in Micrograms per Milliliter (Klett Units)					
	0	20	100	200	400	800
l-Arginine	10	32	69	86	**89**	58
l-Histidine	13	60	**73**	64	56	55
dl-Isoleucine	10	52	65	**71**	58	50
l-Leucine	3	35	62	**79**	69	63
l-Lysine	30	65	73	**83**	72	50
dl-Methionine	12	58	**61**	57	45	45
dl-Phenylalanine	2	50	50	**56**	55	50
dl-Serine	8	51	53	55	**60**	55
dl-Threonine	11	38	47	**57**	54	50
l-Tryptophan	30	62	**65**	60	55	51
dl-Valine	7	37	58	**60**	56	54

* Growth is indicated in Klett readings at the various concentration levels. Optima are shown in boldface type.

TABLE 5

COMPARATIVE AMINO ACID CONCENTRATIONS IN MICROGRAMS PER MILLILITER

Amino Acids	The 100 Series		The 400 Series		The 800 Series Opt. Conc.	Gelatin 0.6 Per Cent	Casein 0.5 Per Cent
	Op. Conc.	×4	Opt. Conc.	×1½			
l-Arginine	100	400	400	600	400	522	167
l-Histidine	75	300	100	150	100	174	110
dl-Isoleucine	25	100	50	75	200	213	213
l-Leucine	25	100	50	75	200	213	213
l-Lysine	25	100	100	150	200	354	277
dl-Methionine	50	200	100	150	100	100	145
dl-Phenylalanine	25	100	100	150	200	84	171
dl-Serine	75	300	200	300	400	132	255
dl-Threonine	50	200	200	300	200	84	176
l-Tryptophan	50	200	100	150	100	100	53
dl-Valine	50	200	100	150	200	200	347
Total		2,200		2,300	2,300	2,276	2,129

about the same for all three series. In addition, the concentrations of the amino acids in a 0.5 per cent casein and a 0.6 per cent gelatin solutions were computed (table from Sahyun, "Amino Acids and Proteins," quoted in Harrow, 1946) and various concentrations, and a comparative series was run, to determine which of the five different combinations of amino acid concentrations supported the best growth. The results are recorded in Figure 2. The 100 series showed the best

growth for the first 48 hours, but after that the 400 series grew as well, if not slightly better, and at 96 hours exceeded all others by a considerable margin. The gelatin and casein concentrations did not support growth as well as the other three.

DISCUSSION

Early attempts to grow *T. geleii* E on special amino acid diets (Elliott, 1935*b*; Hall and Elliott, 1935) were without success for several reasons. Both methionine and threonine, now known to be essential for strain W (Kidder and Dewey, 1945*a*), were not included in the diet because the former was not available and the latter had not been isolated at that time. Various attempts during the ensuing years, with both acid and alkaline hydrolyzed proteins supplemented with single amino acids, resulted in failure. When the essential amino acids for the rat were determined (Rose, 1938), they were also tried on this protozoan without success, probably because several growth factors which are now known to be essential were not included in the diet. It was found that small quantities of yeast extract produced excellent growth in the presence of the amino acids essential for the rat. Undoubtedly, several growth factors, nucleic acid, and perhaps several amino acids, were present in the yeast extract that produced the good growth.

Kline (1943) was able to culture *Colpidium striatum* (probably a strain of *T. geleii*) on a mixture of fifteen amino acids which did not include histidine, leucine, lysine, and threonine. Kidder and Dewey (1945*a*) found that the ten amino acids essential for the rat (arginine, histidine, isoleucine, leucine, lysine, phenylalanine, methionine, threonine, tryptophan, valine) were also essential for strain W. They also found that serine was not essential but highly stimulatory, although it was routinely included in the diet to obtain optimum growth. The results of the present investigation confirm the work of Kidder and Dewey in so far as the ten essential amino acids are concerned; but, in addition, it seems probable that serine is an essential amino acid for strain E. Small quantities of serine do not stimulate growth any more than do small quantities of several other amino acids (see Tables 2, 3, 4).

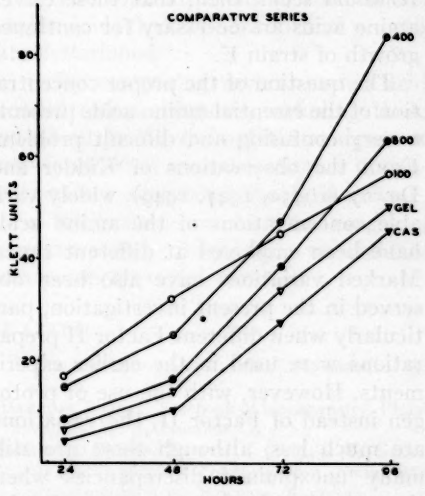

FIG. 2.—Growth is plotted in Klett units against time in hours for the various amino acid concentration mixtures.

In a study of nutritional differences of several strains of *T. geleii* (Kidder and Dewey, 1945*b*) strain E grew only in cultures containing serine, much the same as in the present studies. Furthermore, growth in cultures containing serine but without arginine, isoleucine, phenylalanine, threonine, tryptophan, and valine could be explained by the presence of these amino acids in small quantities as contaminants in the other amino acids, as suggested by the authors, or as constituents of the Factor II preparation. In the present studies growth occurred even after three loop transfers with the omis-

sion of lysine, phenylalanine, threonine, tryptophan, and valine (see Fig. 1). While the Factor II preparations in this investigation were not assayed for amino acids, it was evident that small quantities of the acids were present. When protogen was substituted for Factor II, no growth occurred in any of the cultures upon second transfer when any one of the eleven essential amino acids was omitted. It would seem, then, that these eleven amino acids are necessary for continued growth of strain E.

The question of the proper concentration of the essential amino acids presents a very confusing and difficult problem. From the observations of Kidder and Dewey (1945*a*, 1947, 1949), widely variable concentrations of the amino acids have been employed at different times. Marked variations have also been observed in the present investigation, particularly when different Factor II preparations were used in the earlier experiments. However, with the use of protogen instead of Factor II, the variations are much less, although there are still many unexplained discrepancies when the concentrations are compared in the different series. For example, 10 γ/ml of isoleucine, leucine, and lysine are adequate to support near-maximal growth in the 100 series, while in the 400 series only lysine is so affected. On the other hand, 10 γ/ml of tryptophan in the 400 series supports near-optimal growth, while in the 100 series the same amount permits only half-maximal growth. At higher concentrations small changes have relatively little effect on growth, as is illustrated by arginine in the 800 series, where a change from 200 to 400 γ/ml results in a difference of only 3 Klett units. A possible explanation for these irregularities might be that, as the base amino acid level changes, the interaction between the amino acids likewise changes, so that at low concentrations the amount of the individual amino acids that is available for the organism is highly variable. At higher concentrations this is not so obvious. The results of this investigation seem to indicate that, while, in general, the concentrations should be reasonably close to those shown in the 400 series (Table 5), particularly at the lower levels, there seems to be no sharp, clear-cut level that results in optimal growth.

The optimal amino acid concentrations may vary with other substances in the medium, such as growth factors, carbon sources, and salts. Several series of experiments indicate that the addition of dextrose and sodium acetate made no essential difference in the relative concentrations of the amino acids. There has been insufficient work so far, on both salts and growth factors, to note any effect one way or another.

The principal carbon sources in these experiments are dextrose and sodium acetate. Concentrations of sodium acetate as large as 0.2 per cent are toxic to strain E in casein hydrolysate (Elliott, 1933), although 0.1 per cent concentration in synthetic medium increases growth very effectively. Dextrose has long been used as a source of carbon for this ciliate (Elliott, 1935*a*).

The salts were used in approximately the same concentrations that Kidder and Dewey (1949) found effective for strain W. These workers showed that $CuCl_2$ and $Fe(NH_4)_2(SO_4)_2$ make up a fraction of the original Factor II, known now as "Factor IIB''." Factor IIB′ has been shown to be pyridoxine in high concentrations, and Factor IIA has been demonstrated by Stokstad *et al.* (1949) to be protogen, a growth factor. At first, pyridoxine was thought to be nonessential for strain W, but later it was shown to be essential and that the concentration must be high; it apparently was being supplied

in large quantities with the Factor II preparations (Kidder and Dewey, 1949). All work, so far, with strain E has indicated that pyridoxine is essential for growth. Furthermore, preliminary findings indicate that strain E requires nicotinamide and pantothenic acid, in addition to pyridoxine and protogen for growth. Riboflavin, folic acid, biotin, thiamine, choline chloride, and possibly vitamin B_{12} influence growth but are not essential. By comparison, strain W requires riboflavin and folic acid (Kidder and Dewey, 1949) in addition to the four growth factors required by strain E.

Yeast nucleic acid has been routinely employed in these experiments in the concentration of 100 γ/ml. Its purine and pyrimidine components have been used with equal effectiveness. With the work done so far there is still some question about both the concentration and the exact components of nucleic acid that are essential. This is being investigated at the present time.

SUMMARY

1. Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, and valine are essential for the growth of *T. geleii* E.

2. The concentrations of the various amino acids that support optimal growth are determined.

3. By comparing several amino acid concentration mixtures, including those based on casein and gelatin analyses, it was found that a special mixture of concentrations supported the best growth.

4. While there were optimal ranges of concentrations, there were no definite sharp concentration maxima.

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THE UTILIZATION OF MINUTE FOOD PARTICLES BY GOLDFISH[1]

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ORGANISMS change or "condition" the media in which they live. The conditioning of a few artificially reproducible, habitable types of water by common goldfish has been intensively studied in this laboratory. These fish introduce excretions, secretions, and feces into the water; they also regurgitate food particles following feeding and eject sexual products during the breeding season. We have not worked with water conditioned by breeding fish. Much, but not all, of the increased rate of growth shown by goldfish isolated in homotypically conditioned water, in comparison with appropriate accompanying controls, results from the presence of regurgitated food particles (Allee, Oesting, and Hoskins, 1936; Allee, Finkel, and Hoskins, 1940). Improved growth was also achieved by direct experimental introduction of small particles of food (Livengood, 1937). The series of experiments, the results of which form the basis for the present paper, deals with the possible food value of particles that are smaller than 44 μ in diameter. The method of intake of such minute objects was long unknown, but certain kinds of evidence, which have been confirmed and extended by recent studies (Allee and Frank, 1948), strongly indicate that goldfish regularly swallow water and that suspended colloidal material is swallowed with the water and concentrated in the gut. X-ray examination showed that thorium dioxide in colloidal suspension (Heyden Chemical Corporation's Thorotrast), which is opaque to such rays, becomes concentrated in the intestine but did not give evidence that it collected on mucus in the oral or branchial region of normal fresh-water fishes or that it is otherwise concentrated in that general area.

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ANIMALS AND THEIR TREATMENT

Throughout these tests, common goldfish were employed that ranged from about 3 to 5 cm. in body length. They were obtained from Grassyforks Fisheries, Martinsville, Indiana. Many of the methods described in earlier reports were retained. Body maintenance or growth was determined by measuring changes in length revealed by suitable photographs taken at the beginning and the end of the assay periods. The data are given for changes in length in units of 0.82 mm. Weights were used in some experiments, though not consistently, since they showed no significant differences from trends found by studying changes in length. The methods for obtaining these measurements and for making growth assays have been described elsewhere in detail (see Allee, Bowen, Welty, and Oesting, 1934; Allee, Frank, and Berman, 1946, and citations).

The animals, which were tested once and then discarded, were isolated for

growth assay in 1-gallon earthenware jars containing 1 liter of water. The experiments divide naturally into two series, A and B. The former were primarily exploratory in nature, and in some of them food particles were present in the jars at all times. In such cases, as in all others in both series, the assay fish were transferred to fresh medium in clean jars once a day. In other experiments of this series and in all those in Series B except one, food was added only for the last 3 hours of each 24-hour period. An exception was made in Experiment 8 of Series B, in which introduced bacterial suspensions were present for 15 hours daily. Synthetic pond water (Allee *et al.*, 1934) was the bases for the experimental media in Series A; Chicago tap water with chlorine removed by passing through an activated charcoal filter was so used in Series B. Individual experiments lasted from 20 to 22 days.

This report deals with twenty-five separate tests (Ser. A, ten; Ser. B, fifteen) designed to determine whether these fish are able to derive nourishment from small food particles extending down to colloidal size. In order to yield positive results in a nontoxic medium, the minute particles must (*a*) have food value, (*b*) lack significant toxic effects of their own, (*c*) have no tendency to form larger aggregates that might be assumed to be eaten in a conventional manner, and (*d*) be stable enough not to decompose materially during a feeding period. Available information concerning the mean concentration of food particles present has little real significance, since there was some settling of the large food particles during feeding time. They became somewhat more concentrated near the bottom and more sparse near the surface. We have no evidence that assay fish fed from a scum of food covering the bottom and no full evidence that they did not do so; some fishes in nature browse unclumped diatoms from weed surfaces. Though we were unable to eliminate the increasing density of food suspensions in the lower part of the dishes during the 3-hour feeding period, this does not necessarily mean that aggregated particles were formed. Microscopic examination of the experimental medium showed, at least in Series B, that clumping was not a significant factor.

Trustworthy data are available for the vertical distribution of algal cells after standing for 3 hours. Twenty counts made over a period of 7 days during the progress of one experiment showed a mean concentration of 1,533,000 cells per cubic centimeter in the top few millimeters of water in contrast with 1,912,000 suspended in water near the bottom of the assay jars. The difference has good statistical significance with a P-value of <0.0001.

Series A included preliminary tests of growth or maintenance in suspensions of fresh skim and dried milk, casein and casein derivatives, starch, egg albumen, filtrates and centrifugates from boiled oatmeal, liver, and pulverized dried meat. The filtered oat or meat preparations were made by suspending finely ground rolled oats (or meat) in water, screening the suspension through a phosphobronze sieve with 325 meshes per inch, and then filtering through Whatman No. 3 filter paper. Centrifuged meat or oat suspensions were passed through a Sharples supercentrifuge at 37,000 rpm, then the centrifugate was used. Casein, nutrose (sodium caseinate), albumen, starch, and both fresh and powdered milk were merely suspended in the synthetic pond water. In some experiments casein was treated with dilute NaQH to make it more soluble.

In Series B the experimental food materials consisted of soybean-meal suspen-

sions, bacteria (*Escherichia coli*), either lyophilized or pasteurized, and algal cells (*Chlorella vulgaris*).[2]

Experimental soybean-meal suspensions were prepared by autoclaving the meal and water for 1 hour at 15 pounds pressure per square inch, filtering through Whatman No. 3 paper, centrifuging at 45,000 rpm. The centrifugate was discarded, and the sediment was resuspended. In these particular experiments each fish was exposed to 2 liters of food suspension. The amount of soy meal in suspension was standardized by drying an aliquot and weighing; the concentration was about 100 mg. per liter. The maximum particle size finally obtained was about 5 μ in diameter, although on some days it did not exceed 2.5 μ. A cooked mixture of soybean meal formed the controls for these particular experiments. The lyophilized bacteria were at first prepared by Dr. William Burrows, of the Department of Bacteriology, who also supplied us with cultures; later we lyophilized our own, using his methods. In the other tests with bacteria, *E. coli* were first cultured in a broth medium, passed through the supercentrifuge at 45,000 rpm, resuspended, and pasteurized at 65° C. for 1 hour. They were usually standardized in the same way as the soybean particles, and the suspensions usually had a dry weight of about 25 mg. per liter. The weighed lyophilized bacteria were suspended in a standard amount of water and then added to the experimental jars directly in amounts of 6–25 mg. per liter. In some cases, direct counts in a haemacytometer were attempted; but, because of the Brownian movement and poor visibility of the bacteria, these counts had a large standard error and were not extensively utilized; the numbers recorded varied from 16 to 79 million cells per cubic centimeter in different experiments. The *E. coli* are short bacilli that measure about 1.2 μ in length and 0.8 μ in width.

The algal cells were cultured in 5-gallon carboys in a medium containing per liter 0.5 gm. $NaNO_3$, 0.2 gm. KH_2PO_4, 0.2 gm. $MgSO_4 \cdot 7H_2O$, 0.1 gm. $CaCl_2$, and a trace of $FeSO_4$. From one to three 1,500-watt light bulbs illuminated these containers at various times, usually during the entire 24 hours. CO_2 was bubbled into the carboys for several minutes each day. Initial inoculations were made from previously isolated sterile cultures. Subsequently, sterility was not maintained. Once the *Chlorella* had reached a high concentration, they apparently prevented growth of other algae. Usually 45–50 liters of this culture were centrifuged each day at 35,000 rpm and yielded slightly more than 1 gm. of algae (dry weight). The algae were resuspended in water and their concentration standardized with a haemacytometer. They ranged in diameter from 9 to 12 μ, making this process feasible. The concentration in the assay jars was about 50 mg. per liter dry weight, or between 1.5 and 2 million cells per cubic centimeter.

In the tests of both Series A and B, two types of controls were usually maintained. One consisted of a number of fish treated otherwise in the same way as the experimental fish but receiving no food at all during the 3 weeks of growth assay (starved controls). The other fish were given food suspensions containing particles that obviously could be ingested (fed controls); if possible, this food material consisted of the same substance as the experimental food. In the tests with bacteria and algae, not enough were available, and the fed controls were given a mixture of cooked corn and soybean meal. The nutritive value of algae

[2] Identification by Dr. F. Drouett, Chicago Natural History Museum.

and bacteria was tested separately later: the bacteria and the algae were dried, moistened, and shaped into pellets about 2 mm. in diameter, on the average. These pellets were then introduced directly into the culture water.

For comparative purposes, measurements of the size and number of particles in goldfish-conditioned water were made under a microscope with an eyepiece micrometer. The conditioning coefficient of the water was 100, which is four times the amount of conditioning usually used in experiments reported in previous papers. This coefficient is ascertained by dividing the total length of conditioning fish in millimeters by the number of liters of conditioned water (Allee, Finkel, and Hoskins, 1940).

EXPERIMENTAL RESULTS

The frankly preliminary data from Series A are based on a relatively small number of animals under any given set of conditions. For this reason, regardless of the possible nutritive value of the particles tested, the results are generally inconclusive except in showing that the foods so supplied lack marked growth-promoting power. The kinds of small particles used have been listed, but data from these experiments are not presented in detail. Three types of food—nonsoluble casein, filtered suspensions of oatmeal and of ground liver—each supported slightly positive mean growth in one experiment. In this respect they differed from other experiments using the same materials and from the other foods tried. In Series B, as well as in A, very few of the kinds of small particles tested allowed the fish to get sufficient nutriment to support a gain in weight during the assay period. Substandard feeding or starvation brings first a loss in weight that precedes shrinkage in length. Full maintenance in length and even small increases may be observed when some weight loss has occurred.

In all instances the fish nourished by only minute particles showed a significantly smaller length increase—or usually a decrease—than did the fed controls. These experiments became mainly tests of partial maintenance rather than of positive growth. In most experiments of Series A the mean decrease in length in the experimental fish was only slightly less than that of the starved controls. Three sets of tests in Series A in which the fish averaged significantly better maintenance than those exposed to full starvation for the experimental period are summarized in Table 1, together with three similar summaries from Series B. We have no idea whether any of the other trends suggested in the preliminary experiments might similarly yield statistically significant results, given a large number of repetitions.

Tables 2, 3, and 4 show the experimental and control averages for the individual experiments with each of the three foods tested in Series B. In all experiments but one, the fed control fish showed a slight positive growth. Similarly, the experimental fish as a whole showed less loss in length than did the starved controls. Only in one experiment, and then with algae, did the mean change in length become even slightly positive for the average of the experimental fish, although certain individuals in nearly all the experiments increased slightly in length. Weight changes, which were not always determined, were negative for the experimental fish.

The first three statistically analyzed comparisons shown in Table 1 are calculated, with individual fish as units. In the column labeled *N*, the number to the left gives the size of the experimental population in these cases; that to the right shows the numbers in the respective

controls. Each comparison yields statistically significant differences. In the two individual experiments in which the fish were fed sieved particles of pulverized dried meat the P-values are 0.005 and 0.03, respectively. The experiments with comparisons in this table are based on means for experimental and control populations as units and therefore have a much smaller value for N. The experiments listed in the last two lines of Table 1 were discontinued when the results

TABLE 1

COMPARISON OF CHANGES IN LENGTH IN UNITS OF 0.82 MM. FOR EXPERIMENTAL AND CONTROL FISH

Food Particles	N	Mean of Fed Fish	Mean of Starved Controls	Difference	P
Sieved meat	12:15	−0.32	−0.83	0.51	0.005*
Sieved meat	12:26	−0.21	−0.57	0.36	0.03*
Soybean meal	52:56	−1.42†	−2.02†	0.60	0.001*
Filtered or centrifuged oatmeal	5	−0.07	−0.51	0.44	0.029‡
E. coli	7	−0.39	−0.81	0.42	0.007‡
C. vulgaris	4	−0.36	−0.77	0.41	0.01‡

* Individuals as units; population formula used:

$$t = \frac{\bar{v}_1 - \bar{v}_2}{\sqrt{\frac{(\Sigma v_1^2 - \bar{v}_1\Sigma v_1 + \Sigma v_2^2 - \bar{v}_2\Sigma v_2)(N_1 + N_2)}{(N_1N_2)(N_1 + N_2 - 2)}}},$$

where v represents the datum from one individual.

† Length plus width measurement.

‡ Experiments as units;

$$= \frac{\bar{x}}{\sqrt{\frac{\Sigma x^2 - \bar{x}\Sigma x}{N(N-1)}}},$$

where x is the difference between means from one experiment.

TABLE 2

SUSTENANCE VALUE OF SUSPENSIONS OF SOYBEAN MEAL

Experiment	Food Tested	No. of Fish	Mean Change in Length Plus Width, in Units*
1	Soy-meal control	10	+0.39
	Centrifuged soy meal	20	−1.95
	Starved control	20	−2.34
2	Soy-meal control	35	+0.62
	Centrifuged soy meal	32	−1.09
	Starved control	36	−1.90

* 0.82 mm.

filtered and centrifuged soybean meal involving 52 goldfish exposed to the soy-meal suspension and 56 starved control animals show a difference of about the same order of magnitude with a somewhat more significant P-value. The other showed a fair value of statistical significance; those summarized in the fourth line, being primarily exploratory, were stopped for other reasons. These fish, fed filtered or centrifuged suspensions of oatmeal, shrank over 0.4 mm. less than did

TABLE 3

SUSTENANCE VALUE OF SUSPENSIONS OF BACTERIA

(*Escherichia coli*)

Experiment	Food Tested	No. of Fish	Mean Change in Length, in Units*
1	Corn–soy-meal control	4	+0.56
	Dried *E. coli*	5	−0.87
	Starved control	5	−0.53
2	Corn–soy-meal control	24	+0.06
	Dried *E. coli*	12	−0.40
	Starved control	24	−0.95
3	Corn–soy-meal control	15	+0.20
	Dried *E. coli*	30	−0.57
	Starved control	15	−0.54
4	Corn–soy-meal control	19	+0.34
	Pasteurized *E. coli*	19	−0.06
	Starved control	16	−0.69
5	Corn–soy-meal control	8	+0.39
	Pasteurized *E. coli*	13	−0.19
	Starved control	13	−0.41
6	Corn–soy-meal control	20	+0.90
	Pasteurized *E. coli*	20	−0.54
	Starved control	20	−0.86
7	Corn–soy-meal control	14	+0.44
	Dried *E. coli*	15	−0.14
	Starved control	15	−0.71

* 0.82 mm.

TABLE 4

SUSTENANCE VALUE OF SUSPENSIONS OF ALGAL CELLS

(*Chlorella vulgaris*)

Experiment	Food Tested	No. of Fish	Mean Change in Length, in Units*
1	Corn–soy-meal control	16	−0.56
	C. vulgaris	15	−0.81
	Starved control	16	−1.40
2	Corn–soy-meal control	20	+0.41
	C. vulgaris	20	−0.42
	Starved control	20	−0.88
3	*C. vulgaris*	20	+0.09
	Starved control	20	−0.29
4	*C. vulgaris*	20	−0.31
	Starved control	20	−0.47

* 0.82 mm.

their accompanying starved controls. The difference, despite the small number of paired experiments (5), has a *P*-value of 0.029. The goldfish exposed to suspensions of pure cultures of bacteria with a maximum length of 1.8 μ for the individual cells also showed less shrinkage than did their starved controls; the *P*-value of the difference is 0.007. Exposure to the algae several times larger in diameter (9–12 μ) produced similar results.

In an attempt to determine the intrinsic nutritive value of the algae and bacteria freed from the handicap of their minute size, pellets of these materials about 2 mm. in diameter were fed to some experimental fish. Controls consisted of fish fed the corn–soy-meal mixture and of starved fish. The fish fed pellets of algae grew 0.21 mm. in length during the experimental period. This compares with a mean growth of 0.27 mm. for the controls fed corn-soy meal and with a shrinkage of 0.52 mm. for the starved controls. An appropriate statistical test revealed no significance between the meal-fed and the algae-fed fish, whereas the difference between algae-fed and starved fish had a (statistically significant) *P*-value of 0.014.

The fish fed pellets of lyophilized bacteria grew only 0.08 mm. in length compared with 0.49 mm. for the meal-fed fish and with a decrease in length of 0.49 mm. for the starved fish. Statistical tests show significant differences with a *P*-value of 0.005 for the comparison between meal-fed and bacteria-fed fish and of 0.0004 for the comparison between bacteria-fed and starved fish.

As measured under the microscope, the particles present in water conditioned by goldfish recently fed the corn–soy-meal mixture are predominantly between 5 and 20 μ in diameter. This means that they approximate the size of the *Chlorella* cells used rather than that of *E. coli*. Passing such water through the phosphobronze sieve removed the relatively sparse particles larger than 44 μ in diameter, except for a few short rodlike cylinders molded by the particles passing through the meshes of the screen under slight pressure. Conditioned water did not contain nearly so heavy a concentration of particles from 1 to 12 μ in diameter as did the suspensions of bacteria or of algae. Material smaller than this, even colloidal or dissolved matter, must not be neglected in considering the possible food value of conditioned water, especially since goldfish swallow water together with suspended and dissolved material (Allee and Frank, 1948).

DISCUSSION

The conditions of experimentation were fairly favorable. Comparable results were secured both with synthetic pond water and with dechlorinated city tap water, thereby decidedly lessening the possibility that the positive effects following the conditioning process in these experiments resulted from detoxification of the water (Allee and Bowen, 1932) Among the weaknesses of the analytical work is the fact that we were unable to make completely satisfactory counts of the populations of *E. coli* to which the goldfish were exposed; also we have no reliable data showing the relative amounts of organic material present in conditioned water in gross suspension, in colloidal suspension, and in true solution. An attempt at fractionation into these categories was made with the apparatus available, viz., supercentrifuge, Berkefeld and Seitz filters; but we did not obtain consistent results.

In view of the previously mentioned demonstration that goldfish swallow water containing colloidal particles (Allee and Frank, 1948), the next question concerned the physiological effect produced

by the engulfing of minute food particles and of whatever organic material may be dissolved in the water. Inspection of Tables 2, 3, and 4 shows that goldfish exposed for 3 hours daily to food consisting partly of large particles (fed controls) usually showed a slight mean increase in length during an assay period of 3 weeks. Those exposed to a suspension of smaller particles of oatmeal, soybean meal, dried meat, *E. coli*, or *Chlorella* (Table 1) shrank in length significantly less than did accompanying starved controls. The swallowing of water containing these microscopic particles gave significantly better maintenance than was shown by the temporarily starved fish, although it usually did not produce positive mean growth in length for the experimental populations. The improved maintenance furnishes additional evidence that the particles were ingested and, further, that they provided nourishment.

We have not investigated and have no means of estimating the possible effect of such bacterial or algal populations as we have tested on the maintenance or growth of recently hatched fishes that are just beginning to feed. The ecological importance of such food organisms with such tiny fishes would probably be much greater than with larger individuals.

Pütter (1908, 1909*a*, 1909*b*) thought that many aquatic animals, fish included, could utilize dissolved organic material present in the surrounding water. He postulated nonalimentary (parenteral) feeding with dissolved food intake through the entire epidermis of many animals and in fishes especially through that of the gills. The possibility of nonalimentary feeding needs no extended critical discussion here, particularly as far as fishes are concerned, since (*a*) the evidence is generally negative (Krogh, 1931; Bond, 1933), (*b*) marine fishes have long been known to swallow water in maintaining their osmotic balance (Smith, 1930), and (*c*) recent evidence shows that fresh-water fishes also swallow water. It follows that intake even of dissolved organic material can be explained without furnishing any compulsion toward acceptance of Pütter's proposed nonalimentary intake. Krogh and, to a less extent, Bond have also critically examined the other part of Pütter's theory concerning the nutritive value of organic substances dissolved in water. This subject concerns us somewhat, since the conditioned water used in our experiments with goldfish probably contains dissolved organic material. Pütter (1909*b*) also applied this part of his theory to fishes. Although his estimates of the relative and absolute dissolved organic content of natural bodies of water were too high, such waters do contain more dissolved than particulate organic material (cf. Krogh, 1931; Bond, 1933; Birge and Juday, 1934).

Both Pütter's data and his hypothesis of parenteral food intake in fish nutrition were sharply challenged by Lipschütz (1910, 1913, 1918). In his own experiments with carp, Lipschütz found no indication of the utilization of dissolved organic matter. He categorically denied the validity of Pütter's other results and presented evidence that Pütter's initial premises concerning (*a*) the amount of plankton in the sea, (*b*) the amount of nutriment needed by fish, and (*c*) the amount necessarily present in the gut to account for complete nutrition by particulate food all lacked validity. Lipschütz concluded that, under the conditions tested, fishes can meet their food requirements from particulate food consisting of plankton and detritus or the larger material eaten by predators.

Actually, Knörrich (1901) preceded Pütter by postulating the importance of dissolved nutrients in the cultures of

Daphnia. Wolff (1910) demonstrated the presence of minute organic particles in a filtered sterilized straw infusion such as Knörrich had used and so helped initiate the study of the role of such particles in the nutrition of aquatic animals. Lohmann (1908, 1909, 1911) exerted influence in the same direction by his demonstration of the importance of nannoplankton, including bacteria, in animal nutrition. The significance of nannoplankton in nutrition of larger animals is indicated by the evolution of such elaborate collecting devices as mucus filters by a variety of marine forms, including the echiuroid worm *Urechis* and certain ascidians (Sverdrup, Johnson, and Fleming, 1942).

Krogh (1931) concluded as a result of his own experiments and a review of the literature that there is no convincing evidence proving that any animal, certain protozoans perhaps excepted, gets net nourishment from dissolved organic matter in natural water. Higher aquatic animals may "take up significant quantities of dissolved combustible material, but the quantities excreted are always larger" (p. 440). Tadpoles, mussels, and other animals under experimental conditions can ingest organic matter from somewhat concentrated solutions through the intestine rather than through the integument or gills. They can thrive and grow without particulate food for a considerable time.

Support of such a conclusion is found in the results obtained by Yonge (1928), who studied oysters exposed to glucose solutions. There was no evidence of intake except by way of the alimentary canal or, particularly when the mouth was plugged, by the carrying capacity of leucocytes as they moved through the epithelium back into the body after an excursion into the mantle cavity. Yonge concluded from this and former work that the ciliated epithelium of lamellibranchs can neither absorb food material nor ingest particles phagocytically.

There is good evidence that certain Protozoa, as well as *Ostrea*, *Asterias*, and some amphibian tadpoles, exhibit better maintenance and sometimes even better growth when exposed to sufficiently concentrated solutions of organic nutrients (Krogh, 1931). Kříženecký (1928) extended earlier work with frog tadpoles in fairly high concentrations to solutions of "Bioklein" so diluted in some instances as to furnish nutrition comparable to that in natural habitats. He interpreted his data as meaning that the lower concentrations contributed to maintenance but did not promote growth. Krogh (1931) suggests that obvious variations in Kříženecký's results may mean that "the experimental conditions contain an unknown factor which may have vitiated all the results." We tend toward the milder view that Kříženecký needed more experiments testing the food value of lower concentrations of dissolved nutrients.

Bond (1933) reports that the strain of the ciliate *Colpidium campylum* (=*Tetrahymena geleii*) which he used grows well in the laboratory on dissolved foods provided that a complex source of nitrogen is supplied. He states that "in nature, in forms with like food requirements, it seems possible that a certain part of the utilized food may be taken in a dissolved state" (p. 33). Bond's extended, careful tests with the brine shrimp *Artemia* showed that it is dependent on particulate food. He considers it "extremely unlikely that dissolved organic matter plays any important part in the natural nourishment of the marine zooplankton" (p. 83), except in an indirect course by way of phytoplankton.

Two other cases deserve mention here, although the feeding organisms are far

removed taxonomically from fishes. Perhaps one reason for citing them lies in the fact that in one essential they present opposed results. Gellis and Clarke (1935), with a good knowledge of related preceding inquiries, found that the cladoceran *Daphnia magna* can grow in media containing no particulate matter other than colloids but not in media containing only solutes. Contrariwise, Trager (1936) reported experiments showing that bacteriologically sterile mosquito larvae (*Aedes aegypti*) can be reared to the fourth instar in media that have been ultra-filtered through collodion with the contained nutriment "entirely in true solution."

Trager suggests that particulate substances, even colloidal particles, have a distinct advantage over true solutions as nutriment, since the gut can become sufficiently packed with concentrated particles so that high concentrations of soluble nutrients can be produced by digestion. In contrast, nutrients existing as solutes must enter the gut along with water and are present there in the same concentration as in the surrounding liquid. The possible means for concentrating such dissolved nutrients present different physiological problems from those involved in concentrating particles of colloidal size or larger (Trager, 1936, p. 350).

With regard to the experiments reported in the present paper, it is obviously important to consider the concentration of bacteria and algae in our experiments in relation to that existing in natural habitats. Good quantitative results concerning density of natural populations of aquatic bacteria or unicellular algae are difficult to find in the literature.

ZoBell (1946) says that sea water rarely contains a million bacteria per milliliter. Birge and Juday (1934, p. 451), relying on the direct counts made by Bere (1933), report that the number of bacteria in water from fifty lakes in the Highland Lake District of Wisconsin ranged from a minimum of about 19,000 per cubic centimeter in Diamond Lake, a soft-water lake belonging to the seepage type, to a maximum of 2,000,000 per cubic centimeter in Brazell Lake, which has harder water and belongs to the drainage type. Juday in this same paper estimates that 77,000 bacteria per cubic centimeter, containing about 85 per cent rod-shaped and 15 per cent coccus forms, represented a live weight of 0.104 mg. per liter, 90 per cent of which was water. The dry organic matter approximated 0.0094 mg. per liter.

The bacteria counts by Bere included all visible ones present and are highly useful, even though criticized by Henrici (1939) as not being entirely accurate. We used only quite short bacilli; the larger rodlike forms were included in Bere's counts. The average rod-shaped bacterium from Wisconsin lakes has approximately 36 times the bulk of one spherical in form (Birge and Juday, 1922) and approximately 2.7 times the bulk of a single *E. coli* with its short cylindrical shape.

Our own crude counts of bacterial populations apparently ranging from 16 to 79 million per cubic centimeter[3] are much more concentrated than any apparently authentic estimates we have seen of planktonic bacterial density in natural uncontaminated waters (cf. Henrici and McCoy, 1938; Henrici, 1939). They are also much greater than the densest bacterial populations reported by Hoskins, Ruchhoft, and Williams (1927) from the Chicago Drainage Canal or from the most nearly septic sections of the Des Plaines or Illinois rivers that

[3] Our counts approximate those indicated by comparing dry weights, bacterial population density, and relative bulk of each bacterium with the data given by Birge and Juday (1922, 1934).

carry sewage-laden water from Chicago. The highest counts reported were from samples taken from the Drainage Canal at Lockport, 35 miles from the base point where the Chicago River joins Lake Michigan. The counts reached 3,800,000 per cubic centimeter when plated on gelatin and 4,390,000 per cubic centimeter when agar was used. These are much greater densities than are usually found during the year. The possibility exists that localized high concentrations of bacteria may be much more dense than the generalized population in either uncontaminated or polluted water and that fish might engulf significant amounts of bacteria from such micro-habitat niches. We have no documentation for this suggestion.

Different kinds of bacteria have different values as food for bacteria-feeding animals. The *E. coli* used in these experiments were selected for their small size, nonclumping tendencies as used, and availability, without regard to other qualities, including possible food value. It was known that certain strains of *E. coli* are among the most satisfactory bacteria tested as far as the nutrition of the cladoceran *Moina* is concerned (Stuart, McPherson, and Cooper, 1931) and that another strain of this species was a fairly good food source for the ciliate *Colpidium colpoda* (Burbanck, 1942).

A few of the records regarding high population densities of unicellular aquatic algae follow: (1) *Marine:* Coccolithophoridae, with 5–6 million per liter, gave a milky appearance to the Oslo fjord (Gran, 1912). Diatoms present near beds of the Pismo clam along the California coast in concentrations of about 30,000 per liter; 6×10^8 furnished approximately 1 gm. of dry organic material (Coe, 1947). (2) *Fresh water:* An *Aphanocapsa*, a blue-green alga, with small cells forming colonies mainly of from 15 to 50 cells, had an observed maximum in Lake Mendota of somewhat more than 8 million colonies per liter. The numbers present were fairly large throughout the year. *Cryptomonds*, a green flagellate, yielded 300,000 per liter in November, 1915 (Birge and Juday, 1922). During a diatom epidemic in the Potomac River, a small diatom, *Synedra delicatissima*, showed 5 million cells per liter for a period of 20 days near the intake for the supply of water to the city of Washington (Conger, 1941). The five highest counts of algae (exclusive of the blue-greens) reported by Hasler and Jones (1949) for laboratory ponds ranged between 13 and 39.4 million cells per liter.

In comparison, one set of counts of the *Chlorella* population to which our goldfish were exposed averaged 1,500,000 per cubic centimeter; another averaged 1,700,000 per cubic centimeter (1.5 or 1.7 billion per liter, respectively). This density is much greater than the sample values just given for natural populations. A few of the algal cells were caught on the gills, suggesting mucous adhesion, since the gill rakers are so far apart that they probably did not filter out these *Chlorella* cells. Suggesting that the algal spheres are captured by mucus raises the possibility that colloidal particles should also be collected in this way, but we have found no X-ray evidence of such collection of colloidal thorium dioxide on the gills of goldfish (Allee and Frank, 1948) or of any healthy native fishes tested (unpublished). Neither have we found by inspection of the surface any indication of ciliated epithelium in the mouth or pharynx of goldfish that might transfer mucus-caught particles to the gut. (No transverse sections were made.) The description in Krause (1923) of the microscopic anatomy of the mouth region of *Esox lucius* does not suggest the presence

of oral cilia. This evidence is admissible here, since unpublished X-ray tests have shown that *E. vermiculatus* engulfs colloidal suspensions of thorium dioxide apparently much as goldfish do. The lack of evidence of the presence of oral cilia in these fishes lends further support for the idea that fresh-water fishes swallow water and any food particles it may contain.

We have no good evidence concerning the rate at which any fresh-water fish swallows water, and obviously a careful determination of this point needs to be made. Also, as far as method of food intake is concerned, we are not entirely convinced that the fish did not feed to some slight extent on the thin film of algae that settled to the bottom, but we have no evidence, behavioral or otherwise, that such feeding occurred.

SUMMARY

1. Goldfish starve less rapidly if allowed to swim for 3 hours daily in water containing suspensions of the bacterium *E. coli* (*ca.* 16–79 million per cubic centimeter; dry weight, 6–25 mg. per liter) or of the green alga *C. vulgaris* (1.5–2 million cells per cubic centimeter, making about 50 mg. per liter dry weight) than if totally starved.

2. Pellets of the alga support goldfish growth better than do pellets of similar size made from this species of bacteria. Fed so, both promoted increases in mean length.

3. Maintenance of length is comparable in these bacterial or algal suspensions to that given by similar exposure to somewhat more concentrated suspensions of soy meal with particles mainly intermediate in size between *Escherichia* and *Chlorella* and to particles of pulverized dried meat or to filtered or centrifuged suspensions of oatmeal (see Table 1).

4. The experimental concentrations of bacteria and algae are more dense than those normally occurring in suspension in natural bodies of unpolluted water.

5. Rough exploratory experiments gave no clear evidence of better maintenance when goldfish were exposed to suspensions of fresh or dried skim milk, casein, casein derivates, starch both soluble and nonsoluble, egg albumen, and liver particles.

6. In other preliminary experiments the fish showed somewhat better maintenance in filtered or centrifuged suspensions of ground oatmeal and of pulverized dried meat than in accompanying starved controls.

7. These results support earlier indications that goldfish under experimental conditions can extract useful food when sufficient concentrations of small particles are present.

8. Certain relations with Pütter's theory of the nutrition of aquatic animals are discussed.

9. Presumably the suspended particles are swallowed along with the containing water, as has already been found for goldfish swimming in suspensions of colloidal thorium dioxide.

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APPLICATION OF THE CONVERSE BERGMANN PRINCIPLE TO THE CARABID BEETLE, DICAELUS PURPURATUS

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THIS study began in a casual way when it was noted that specimens of the conspicuous purple carabid, *Dicaelus purpuratus* Bonelli, collected in southern Louisiana were larger than those collected near the southern end of Lake Michigan.

With the accumulation of more material, it was found that (1) the change in average size of individuals of the population appeared to be gradual and uniform from more northern to more southern areas within the range of the species; (2) such a change was postulated by that portion of the Bergmann zoögeographic principle relating to poikilothermal terrestrial animals; (3) very little firsthand data had been accumulated by American biologists with reference to the Bergmann principle.

It will be remembered that this principle states that homoiotherms tend to be larger in colder regions than in warmer regions within the same species population. This change in size may be explained partially by the physiological datum that heat radiation is said to be about the same per unit of skin surface under identical conditions, and partially by the Spencerian doctrine that volume increases as the cube of linear dimensions, whereas surface increases as the square of these dimensions. Consequently, the larger the homoiotherm, the less disadvantageous the lower air temperature of colder portions of its range. The smaller homoiotherms have a smaller total surface area, but this area is relatively larger in relation to volume, so that heat loss by radiation is relatively greater than for larger animals. Conversely, poikilotherms tend to be smaller in colder, and larger in warmer, regions within the same population. In general, the Bergmann principle is rather well known, but very little specific use has been made of it in the distribution of North American insects. A more complete discussion of this principle, and of exceptions to it, may be found in Rensch (1936, 1938, 1939), Hesse, Allee, and Schmidt (1937), and Allee, Emerson, Park, Park, and Schmidt (1949).

Dicaelus purpuratus lends itself very well to such a zoögeographic analysis. In the first place, it is relatively both large and conspicuous. Adults average about 1 inch in length. Most specimens are a deep purple on head, pronotum, and elytra; the legs, labial palpi, maxillary palpi, and first three antennal segments are slightly more reddish-purple; the tarsal claws, setae, distal surface of palpi and fourth to eleventh antennal segments are yellowish-brown. The general purple color changes in tone with direction of light beam. Specimens tend to become covered with a film that darkens the surface in pinned specimens, so that an oil solvent must be used to clean them if the true color of the cuticle is desired. This large size and coloration usually assist in the detection and collection of the species and hence assure that museums and private collections are likely to have a series available for study.

Second, the species is not easily confused with its congeners. The most close-

ly related, *D. splendidus* Say, is much more highly colored, with a distinct golden bronze or cupreous integumental color.

Third, *purpuratus* is sexed with ease. The males have the prothoracic tarsi dilated and partially spongiose-pubescent beneath; the females have these tarsi undilated and not spongiose beneath.

Finally, the range of the species is extensive. In general, *purpuratus* is distributed over the eastern half of the United States from about 71°00′ to 93°00′ west longitude and from about 29°30′ to 43°00′ north latitude. Leng (1920) gives the range as "New York–Florida-Missouri-Louisiana." It is with the range in latitude that we are concerned, the species being dispersed through approximately 14°. The general distribution pattern is suggested by parallel lines, and localities from which specimens were studied are indicated by dots in Figure 4.

Since the purpose of this investigation was primarily to discover whether or not this species of beetle could be used to demonstrate that part of the Bergmann principle that applied to poikilothermal animals, specimens were measured as follows: The length of a beetle was taken by a pair of dividers from the apical margin of the labrum to the apical limit of the elytra, and the greatest width of the specimen was obtained with the same instrument. It was found that the females were slightly wider relatively than the males as a rule. Since the sex ratio was 1:1 in the sample examined, this slightly greater width of females was spread through the population and did not alter the general conclusion. Consequently, sex of the specimen was not considered in this preliminary body-size–latitude analysis.

On the other hand, both length and width, taken together, gave a single figure that could be plotted. Considering the beetle as a rectangle, length multiplied by greatest width gave "dorsal area." Obviously, this method gave a greater area than the true dorsal area by as much as 25 per cent or more, since it did not allow for the tapered anterior and posterior ends of the body. The alternative, of attempting to allow for curvatures of the lateral outlines, was abandoned as too full of error for statistical purposes.

In Table 1 data are given upon which this report is based, namely, length and width in millimeters, dorsal area in square millimeters, collecting localities, collections in which the specimens are deposited, and the approximate location of the collecting localities in terms of north latitude.

In this table the column "Approximate Locality" contains the localities as given on specimen labels, whereas the last column attempts to give the latitude of the actual collecting site, often as much as 15 miles from the nearest post-office address.

Some of these data are unsatisfactory. Not included are specimens collected many years ago bearing such labels as "So. U.S.," "Pennsylvania," "North America," and so on. In other cases material bearing state labels could be used in a general consideration of areal range but were not used in plotting distribution curves, as, for example, two specimens labeled "Massachusetts" (Illinois Natural History Survey), and one from "Wisconsin" and three from "Michigan" (Chicago Natural History Museum).

In several cases the latitude could not be given for certain collecting localities and had to be estimated, as, for example, "Saraland" ("Baraland"?), Alabama.

Abnormal specimens, which included what were apparently the results of injuries during the pupal period, such as asymmetrical pronotum and one elytron

TABLE 1

Approximate Locality	Collection*	Length (Mm.)	Width (Mm.)	"Dorsal Area" (Sq. Mm.)	North Latitude
Asheville, N.C.	Chicago Natural History Museum	24.5	9.5	232.75	35° 30′
Half Day, Ill.	Chicago Natural History Museum	23.0	8.5	195.50	41 40
Half Day, Ill.	Chicago Natural History Museum	21.0	8.5	178.50	41 40
Willow Springs, Ill.	Chicago Natural History Museum	20.0	8.0	160.00	41 32
Palos Park, Ill.	Chicago Natural History Museum	21.0	8.5	178.50	41 30
Olive Branch, Ill.	Chicago Natural History Museum	26.0	10.0	260.00	37 00
Olive Branch, Ill.	Chicago Natural History Museum	24.0	9.5	228.00	37 00
Olive Branch, Ill.	Chicago Natural History Museum	24.0	9.0	216.00	37 00
Olive Branch, Ill.	Chicago Natural History Museum	24.0	9.5	228.00	37 00
Olive Branch, Ill.	Chicago Natural History Museum	23.5	9.0	211.50	37 00
Olive Branch, Ill.	Chicago Natural History Museum	22.5	9.0	202.50	37 00
Olive Branch, Ill.	Chicago Natural History Museum	23.5	9.0	211.50	37 00
Olive Branch, Ill.	Chicago Natural History Museum	23.5	9.0	211.50	37 00
Joliet, Ill.	Orlando Park	21.0	8.0	168.00	41 32
Springfield, Ill.	Orlando Park	21.0	8.5	178.50	39 45
Springfield, Ill.	Orlando Park	22.5	9.0	202.50	39 45
Turkey Run, Ind.	Orlando Park	22.0	9.0	198.00	39 50
Tremont, Ind.	Orlando Park	21.5	8.5	182.75	41 40
Urbana, Ill.	Orlando Park	21.0	8.0	168.00	40 10
Urbana, Ill.	Orlando Park	22.0	8.5	187.00	40 10
Urbana, Ill.	Orlando Park	23.0	8.5	195.50	40 10
Urbana, Ill.	Orlando Park	23.0	9.0	207.00	40 10
Urbana, Ill.	Orlando Park	24.0	9.5	228.00	40 10
Urbana, Ill.	Orlando Park	23.0	9.0	207.00	40 10
Urbana, Ill.	Orlando Park	23.0	9.0	207.00	40 10
Urbana, Ill.	Orlando Park	22.0	8.5	187.00	40 10
Urbana, Ill.	Orlando Park	23.5	9.0	211.50	40 10
Peoria, Ill.	Orlando Park	22.0	9.0	198.00	40 45
Myrtle Grove, La.	Orlando Park	28.0	10.0	280.00	29 30
Smith, Ind.	Orlando Park	23.0	9.5	221.50	41 40
Smith, Ind.	Orlando Park	24.0	10.0	240.00	41 40
Smith, Ind.	H. F. Strohecker	24.5	9.0	220.50	41 40
Milwaukee, Wis.	Milwaukee Public Museum	21.0	9.0	189.00	43 00
Richmond, Ind.	T. J. Daggy	20.8	8.4	174.72	39 50
Richmond, Ind.	T. J. Daggy	20.8	8.4	174.72	39 50
Smith, Ind.	T. J. Daggy	22.0	9.6	211.12	41 40
Galesburg, Ill.	Illinois Natural History Survey	21.0	8.0	168.00	40 55
Villa Ridge, Ill.	Illinois Natural History Survey	24.5	9.5	232.75	37 10
Turkey Run, Ind.	Charles Seevers	20.8	8.0	166.40	39 50
Turkey Run, Ind.	Charles Seevers	20.8	8.5	176.80	39 50
Michigan City, Ind.	Charles Seevers	21.8	8.6	187.48	41 43
Smith, Ind.	Charles Seevers	23.0	9.0	207.00	41 40
Smokemont, N.C.	Charles Seevers	25.0	9.6	240.00	35 35
Greenbrier, Smoky Mts., Tenn.	Charles Seevers	25.0	10.2	255.50	35 55
Hocking County, Ohio.	Ohio State Museum	22.5	9.5	213.70	39 30
Hocking County, Ohio.	Ohio State Museum	22.0	9.0	198.00	39 30
Hocking County, Ohio.	Ohio State Museum	20.5	9.0	184.50	39 30
Hocking County, Ohio.	Ohio State Museum	23.5	9.5	223.20	39 30
Hocking County, Ohio.	Ohio State Museum	22.0	8.6	198.20	39 30
Fernbank, Ohio.	Ohio State Museum	24.0	9.5	228.00	39 06
Cincinnati, Ohio.	Ohio State Museum	21.7	9.0	195.30	39 06
Cincinnati, Ohio.	Ohio State Museum	24.0	9.0	216.00	39 06
Scioto County, Ohio.	Ohio State Museum	24.0	9.7	232.80	38 45
Adams County, Ohio.	Ohio State Museum	20.8	8.4	174.70	38 45
Adams County, Ohio.	Ohio State Museum	22.3	9.2	205.10	38 45
Adams County, Ohio.	Ohio State Museum	24.5	9.5	232.70	38 45
Franklin County, Ohio.	Ohio State Museum	22.6	9.0	203.40	39 55
Detroit, Mich.	Ohio State Museum	23.5	9.5	223.20	42 15
Tyrone, Ky.	Ohio State Museum	22.0	8.6	189.20	38 07

* Gratitude is expressed to the following for permission to study specimens under their care: Mr. W. J. Gerhard and Mr. Rupert Wenzel (Chicago Natural History Museum); Dr. H. F. Strohecker (University of Miami, Coral Gables, Fla.); Mr. Herrl (Milwaukee Public Museum); Dr. Tom Daggy (Davidson College, Davidson, N.C.); Dr. T. H. Frison and Dr. H. H. Ross (Illinois Natural History Survey); Dr. Charles Seevers (Roosevelt College, Chicago); Mr. E. S. Thomas and Dr. C. H. Kennedy (Ohio State University); Dr. Noland (University of Wisconsin).

TABLE 1—*Continued*

Approximate Locality	Collection*	Length (Mm.)	Width (Mm.)	"Dorsal Area" (Sq.Mm.)	North Latitude
Cincinnati, Ohio	Wenzel Collection, Ohio State Univ.	22.8	9.0	205.20	39° 06′
Cincinnati, Ohio	Wenzel Collection, Ohio State Univ.	22.0	8.8	193.60	39 06
Hanging Rock, Ohio	Wenzel Collection, Ohio State Univ.	25.0	10.0	250.00	38 40
Jefferson, Ohio	Wenzel Collection, Ohio State Univ.	22.0	8.5	187.00	41 45
Jefferson, Ohio	Wenzel Collection, Ohio State Univ.	23.0	9.1	209.30	41 45
Jefferson, Ohio	Wenzel Collection, Ohio State Univ.	23.0	9.0	207.00	41 45
Shelby County, Ohio	Wenzel Collection, Ohio State Univ.	23.5	9.2	216.00	40 20
Orange, N.J.	Wenzel Collection, Ohio State Univ.	25.5	9.8	249.90	40 45
Orange, N.J.	Wenzel Collection, Ohio State Univ.	23.5	9.0	211.50	40 45
Orange, N.J.	Wenzel Collection, Ohio State Univ.	24.0	9.3	223.20	40 45
Orange, N.J.	Wenzel Collection, Ohio State Univ.	22.5	8.5	191.20	40 45
Black Mts., N.C.	Wenzel Collection, Ohio State Univ.	24.5	9.2	225.40	35 30
Wilmington, N.C.?	Wenzel Collection, Ohio State Univ.	21.0	8.0	168.00	34 15?
Wilmington, N.C.?	Wenzel Collection, Ohio State Univ.	20.5	8.0	164.00	34 15?
Pittsburgh, Pa.	Wenzel Collection, Ohio State Univ.	22.5	9.0	202.50	40 26
Jeannette, Pa.	Wenzel Collection, Ohio State Univ.	22.5	8.8	198.00	40 20
Jeannette, Pa.	Wenzel Collection, Ohio State Univ.	21.5	8.2	176.30	40 20
Delchamps, Ala.	Wenzel Collection, Ohio State Univ.	25.0	9.5	237.50	30 24
Delchamps, Ala.	Wenzel Collection, Ohio State Univ.	25.0	10.0	250.00	30 24
Saraland, Ala.	Wenzel Collection, Ohio State Univ.	29.5	11.0	324.50	30 30?
Somers Point, N.J.	Wenzel Collection, Ohio State Univ.	25.5	9.3	237.10	39 17
New Orleans, La.	University of Wisconsin	26.0	11.3	293.80	30 00
"Maryland, 1874"	University of Wisconsin	22.5	9.0	202.50	39 35?

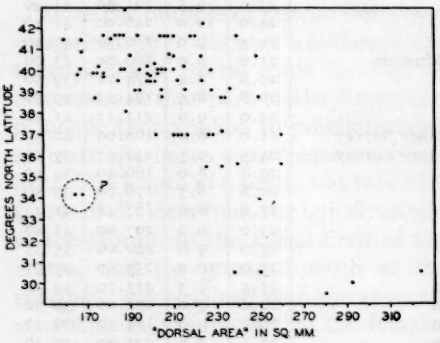

FIG. 1.—Increase in size of adult *D. purpuratus* from northern to southern portions of its range. Each dot represents a specimen measured.

much shorter than the other, were omitted from the calculations. Also several specimens were dwarfs, that is, were much smaller than the minimum for the population as a whole. Two of these had elytral abnormalities as well. Such individuals were very uncommon in general, but dwarfs were examined from as far north as southern Wisconsin and as far south as southern Alabama.

On the other hand, two specimens from Wilmington, North Carolina (Table 1, Fig. 1) were normal in every way, but very small (smaller than some abnormal or dwarfed individuals seen). These two North Carolina spec mens are exceptions to the Bergmann principle, even taking altitude into account, and the author has no satisfactory explanation for them, unless insufficient food supply during the larval period is involved.

Finally, two specimens from "Detroit, Florida" (Ohio State University, Wenzel Collection), are not included, as they may represent either a new species of *Dicaelus* or a new subspecies of *purpuratus*. These specimens are purplish in color but have the third, fifth, and seventh elytral intervals strongly elevated.

In all, ninety-nine specimens of *purpuratus* were studied, and, of these,

eighty-two were found normal and with sufficiently complete locality data to be used (Table 1).

The author would like to criticize his data from two points of view: first, the sample is too small; second, no effect of altitude has been considered in the material to follow. With respect to the first point, there is not much hope that a really large sample, in the statistical sense, can be obtained. It has been estimated that there are available for study about 250 specimens of *purpuratus* in American museums. Of these, about one-third have been examined. In a future report it is hoped that all the others can be used, but, even so, the total number will not be so large as desired. Especially needed is material in this species from Mississippi, Alabama, Georgia, and South Carolina, since the Bergmann curves are incomplete from latitude 32° to 34°.

With respect to the second objection, no good data on the altitude at which particular specimens were collected existed in the material examined. This is a serious fault, since altitude should compensate for latitude in the application of the Bergmann principle to poikilotherms. It is hoped that this gap can be filled, since arrangements are being made to check elevation in the Smoky Mountains–Black Mountains region.

The "dorsal area" for these eighty-two specimens is plotted against degrees of north latitude in Figure 1. With the exception of the two anomalies from Wilmington, North Carolina, it will be seen that there is a general increase in size as the southern limit of the range is approached. Furthermore, as gaps in the series are filled, there is a tendency for this correlation to become more pronounced. For example, this general picture was reported on some years ago (Park, 1939), and in the interval about 50 per cent more specimens have been measured, but the general trend has remained unchanged. Body-length can be plotted in the same way, and the positive correlation between larger size and southward distribution is equally convincing.

The fragment of the *purpuratus* population studied showed a gratifying range in size. The smallest specimen was 8 mm. wide and 20.0 mm. long; the largest measured 11.0 mm. in width by 29.5 mm. in length. The range in dorsal area was from 160.0 to 324.5 sq. mm. In other words, the over-all variation in length was 32 per cent, in greatest width (one specimen measured 11.3 mm. but was shorter than the largest specimen) was 29 per cent, and the variation in "dorsal area" was about 56 per cent.

It should be emphasized not only that an evaluation of the Bergmann principle as it applies to poikilotherms should involve as large a sample of normal individuals as possible, over as great a distance in latitude or altitude as possible, but also that the average for a particular latitude or altitude rather than particular individuals is critical. With this in mind, the several population samples for each 2° of latitude were examined in terms of "dorsal area." The results are given in Table 2.

Of the eighty specimens, not including the two noted previously, it will be seen that the minimum size, maximum size, and average of the population sample all increase from north to south. This is especially well shown in graph form (Fig. 2), in which the average of the whole sample studied (208.87 ± 3.65 sq. mm.), as well as the several latitude classes, are given. In this figure the importance of the average rather than a particular individual is obvious. For example, the smallest specimen in the most southern sample is smaller than the largest speci-

men in the most northern sample, but the averages of the samples are larger toward the southern limit of the species.

Within the limits of the material studied, it is interesting to see how closely the obtained averages are correlated with the calculated averages. The difference between the average of the 41°–43° class and the average of the 29°–31° class is 80.81 sq. mm., or 13.46 sq. mm. average increment for every 2° of north latitude from northern to southern limits of the areal range. When these calculated averages are plotted with the averages obtained by measuring the population samples available (Fig. 3), the congruence is convincing, despite the relatively few specimens involved.

The data upon which this paper was written (Table 1) were given a number of statistical checks.[1] These included the application of the "Student" t test for probability.

TABLE 2

Latitude Class	No. of Specimens Available	Minimum Size (Sq. Mm.)	Maximum Size (Sq. Mm.)	Average Size (Sq. Mm.)
43°–41°	17	160.00	240.00	198.02
41–39	39	168.00	249.90	199.93
39–37	7	174.70	250.00	216.75
37–35	12	202.50	260.00	226.88
35–33	0	.....	.....	.....
33–31	0	.....	.....	.....
31–29	5	237.50	324.50	277.16

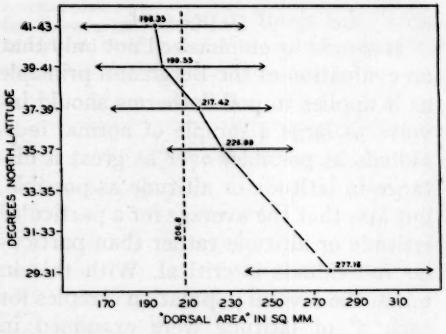

FIG. 2.—Increase in size of adult *D. purpuratus* from northern to southern portions of its range in terms of population samples studied for every 2° of north latitude. Arrows indicate range in sample dorsal area, dots are averages for the sample, and the average of all specimens studied is indicated as a vertical dotted line. The figure was drawn prior to the statistical check, and the slight differences in the sample averages for the first three latitude classes in the figure and as given in Table 2 are the results of reallocating six specimens in these three samples.

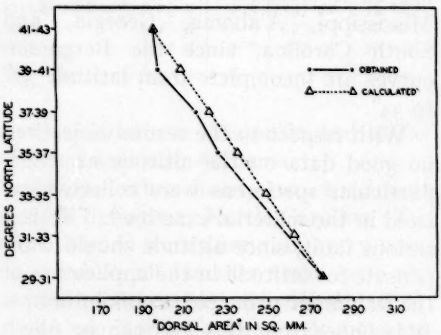

FIG. 3.—Relation of obtained to calculated averages, for every 2° of north latitude, in the increase in size of adult *D. purpuratus* from north to south.

The calculations in Table 3 are based on the formula

$$t = \frac{c - o}{\sigma}\sqrt{n - 1},$$

[1] The author thanks Mr. Richard Edgren, of Cresap Biological Laboratory, and Dr. Mason Wescott, of the department of mathematics, Northwestern University, for the statistical analysis of the data.

in which + equals a p-value of less than 0.01 and in which the mean values (c and o) are rounded off to the nearest whole number. As the samples from the various segments of the population studied were quite small, many inaccuracies would have been introduced by a calculated probability from the normal curve, and the "Student" t test was used. This test compares one set of data with the mean of another set. Table 3 is to be read as follows, taking the comparison of the 31°–33° north latitude class with the 29°–31° north latitude class as an example: the probability of taking five random specimens with a mean dorsal area of 277 sq. mm. and a standard deviation of 12.5 sq. mm. from a universe with a mean dorsal area of 265 sq. mm. is 0.15. That is, the chances of finding five specimens in the 29°–31° class with a mean dorsal area of 277 sq. mm., σ of 12.5 sq. mm., is 15 per cent, or roughly one in six. Here the chances are fairly good, but not extremely high, even in the two adjacent latitude classes. It will be noted from this table that the smaller the divergence in latitude, the greater the probability that chance alone could explain the divergence in size. In all cases a latitude difference of 4° produces highly significant p-values (less than 0.01), or, in other words, the probability is less than one in one hundred. In most of these latitude comparisons, the divergence is so great that t-values cannot be computed from available tables.

TABLE 3

"STUDENT" t TEST FOR PROBABILITY

Latitude Class	29°–31°						31°–33°						33°–35°					
	c	o	σ	n	t	p	c	o	σ	n	t	p	c	o	σ	n	t	p
29°–31°	....	....	....	....	.....	.....	265	277	12.5	5	− 1.92	0.15	249	277	12.5	5	− 4.48	+
31°–33°	277	265	.....	0	.....	.....	.....	.....	.....	.....	.....	.....	249	265	.....	0	.....	.....
33°–35°	277	249	.....	0	.....	.....	265	249	.....	0	.....	.....	.....	.....	.....	.....	.....	.....
35°–37°	277	227	20.8	12	7.97	+	265	227	20.8	12	6.06	+	249	227	20.8	12	3.51	+
37°–39°	277	217	23.4	7	6.28	+	265	217	23.4	7	5.02	+	249	217	23.4	7	3.35	0.14
39°–41°	277	200	20.8	39	22.9	+	265	200	20.8	39	19.3	+	249	200	20.8	39	14.5	+
41°–43°	277	198	21.1	17	14.9	+	265	198	21.1	17	12.7	+	249	198	21.1	17	9.7	+

Latitude Class	35°–37°						37°–39°						39°–41°					
	c	o	σ	n	t	p	c	o	σ	n	t	p	c	o	σ	n	t	p
29°–31°	227	277	12.5	5	− 8	+	217	277	12.5	5	− 9.6	+	200	277	12.5	5	−12.32	+
31°–33°	227	265	.....	0	.....	.....	217	265	.....	0	.....	.....	200	265	.....	0	.....	.....
33°–35°	227	249	.....	0	.....	.....	217	249	.....	0	.....	.....	200	249	.....	0	.....	.....
35°–37°	....	....	....	....	.....	.....	217	227	20.8	12	− 1.59	.15	200	227	20.8	12	− 4.31	+
37°–39°	227	217	23.4	7	1.05	0.36	....	....	....	....	.....	.....	200	217	23.4	7	− 1.78	0.16
39°–41°	227	200	20.8	39	8.01	+	217	200	20.8	39	5.5	+	....	....	....	....	.....	.....
41°–43°	227	198	21.1	17	5.5	+	217	198	21.1	17	3.61	+	200	198	21.1	17	0.379	0.61

Latitude Class	41°–43°					
	c	o	σ	n	t	p
29°–31°	198	277	12.5	5	−12.64	+
31°–33°	198	265	.....	0	.....	.....
33°–35°	198	249	.....	0	.....	.....
35°–37°	198	227	20.8	12	− 4.63	+
37°–39°	198	217	23.4	7	− 1.99	0.10
39°–41°	198	200	20.8	39	− 0.594	0.56
41°–43°	....	....	....	....	.....	.....

On the basis of the data studied, then, *D. purpuratus* conforms with that part of the Bergmann principle that is concerned with poikilothermal animals. The explanation is not readily apparent. In

the first place, since the adult beetle does not increase in size, once the sclerotization of its cuticle is completed, most of the burden of explanation for this increase in adult size from north to south is placed upon ecological and physiological factors of the larval and pupal periods. This paradoxical situation is unfortunate, since much more is known about the postpupal life of insects than about their developmental periods.

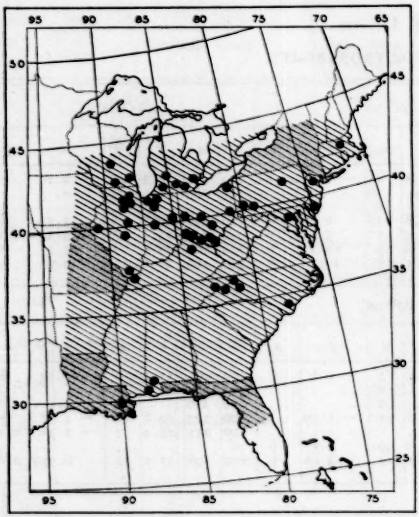

FIG. 4.—Distribution of the carabid beetle, *D. purpuratus*. Dots represent localities from which one or more specimens were studied. States stippled are those listed by Leng (1920) as peripheral areas of the range. Diagonal lines suggest the probable area of dispersal.

Nevertheless, since the success of the species depends upon all stages in the life-cycle, the adults are indirectly important in this special question, and all available facts are desirable if a tentative explanation is to be reached.

Several points need examination. One involves the community-type inhabited. A glance at the general distribution of *purpuratus* (Fig. 4) is sufficient to show that this species ranges within the deciduous forest biome of eastern North America. In fact, this carabid may be used as a faunal index of this region, but its exact habitat niche may be more narrowly limited. The author has collected individuals of this species at intervals during the last twenty-five years. Examination of the notebook reveals that in Illinois *purpuratus* is found in red oak–burr oak–sugar maple forests or in nearly pure stands of maple; eastward into Indiana, it is found in beech–sugar maple forests; southward, the species is associated with tulip forests at lower altitudes, and in southern Louisiana it is found in live oak–sweet gum–Spanish "moss" forests. It is not found usually in pioneer communities or in areas greatly disturbed by man. Consequently, *purpuratus* may be included in the list of animals that are characteristic of rich deciduous woods, probably an index of climax deciduous forest over its range. Inferentially, the species may be expected to become less common. It appears, then, that the population is very unevenly distributed through its range but that this dispersal pattern is related to the distribution of rich, mature, climax forests.

Within such forests the habitat of *purpuratus* is the floor stratum. By day these beetles occupy niches beneath fallen logs or under the bark of decaying logs. As with the majority of Carabidae (Park, 1940), this species is nocturnal. It leaves its niches during the dusk period in search of food. In the field the beetle has been observed in activity between 6:00 P.M. and 10:00 P.M. on several occasions. This nocturnal activity has been observed by the author only in the northwestern portion of its range. Krumbiegel (1932) studied the activity of *Carabus nemoralis* Müller under experimental conditions. This latter species ranges in Europe from Ireland into European Rus-

sia and from southern Spain and northern Italy into central Norway. Krumbiegel found that, in general, individuals of *nemoralis* from the northern parts of its range were darker in color, photonegative, and nocturnal, whereas those from southern parts of the range were lighter in color, photopositive, and diurnal in activity. This species has been introduced into the United States and reached the Chicago area some years ago (Park, 1929); but not enough is known about its penetration and colonization of the southern United States to repeat the experiments of Krumbiegel. On the other hand, *D. purpuratus* has a known range of nearly 14° of latitude and should make an interesting check of the Krumbiegel conclusions with a different species in the same family.

Not only is *purpuratus* nocturnal where reported under field conditions, but it is nocturnal under experimental conditions. This is not the occasion for an analysis of its activity pattern, but an example will be helpful. Using the balanced-disk electrical recorder described previously (Park, 1935), individuals of *purpuratus* were found to maintain their nocturnal pattern of activity for three consecutive nights in constant darkness, constant air temperature, and constant rate of evaporation. An example is given in Figure 5.

Another aspect of species ecology that may have a bearing on the success of the population is the food and feeding habits of the adults. The *purpuratus* observed were wholly carnivorous. In the laboratory they fed on both beef flesh and juice. In the field the beetles were predaceous. One was observed to kill and devour an individual of the tenebrionid, *Scotobates calcaratus* (Fab.), leaving only the legs and elytra, between 6:00 P.M. and 9:00 P.M. More frequently, *purpuratus* attacked snails. Large snails, such as *Polygyra thyroidus* (Say), were observed in relation to the beetle's method of feeding. Each time that the snail protruded its head or foot, the beetle would dart at the exposed area, pounce, and bite into the flesh. The snail would withdraw its body, and the beetle would remain near the aperture of the shell in an oblique crouch. When the victim's head or foot reappeared, the attack was repeated. Eventually the snail would be killed, and

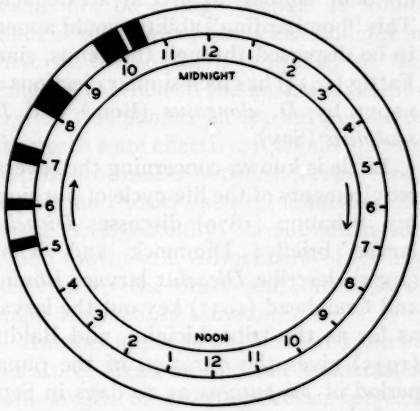

FIG. 5.—Clock diagram of the activity of a single *D. purpuratus* from May 27 to May 29, 1932, in constant darkness, with cabinet temperature held between 24°.2 and 24°.6 C. and rate of evaporation at 0.3 cc. per hour. Black bands denote periods of activity.

the beetle might eat from half to two-thirds of the body during the night. These field observations agree with the scanty data in the literature. Liebeck (1890) noted that *D. dilatatus* Say fed upon the snail, *Anguispira alternata* (Say) while the latter was in its shell, and Forbes (1883) dissected *Dicaelus* and found only fluid animal food in its digestive tract.

Although *purpuratus* feeds upon other animals, there appears to be a tendency for this beetle to feed upon snails, especially *Polygyra*. This tendency coincides with the habitat of both predator and

prey, namely, floors of rich deciduous forests. Furthermore, there is a general coincidence between the period of activity of the food-animal and the feeder.

The enemies of *purpuratus* probably include toads and insectivorous birds and mammals of the forest. At least one method of defense may be the "bombarding" behavior of these beetles. They have been seen suddenly to eject a misty vapor, probably from anal glands, that has an odor similar to glacial acetic acid. This "bombarding" ability would appear to be dispersed through the genus, since Fattig (1933) has seen similar ejections of vapor by *D. elongatus* (Bon.) and *D. sculptilis* (Say).

Little is known concerning the several requirements of the life-cycle of *purpuratus*. Schaupp (1879) discusses *Dicaelus* larvae briefly; Dimmock and Knab (1904) describe *Dicaelus* larvae; Böving and Craighead (1931) key out the larvae as far as the tribe Licinini, and Balduf (1935) gives the duration of the pupal period of *purpuratus* as 10 days in September.

The author has accumulated a few more data on the life-history of this species, and these may be summarized. In northern Illinois *purpuratus* hibernates as an imago from about the middle of October to the end of March. The hibernaculum is usually in well-decomposed log mold or in leaf mold beneath rotting logs. Copulation, oviposition, and early larval instars have not been seen, but a last instar larva was collected at 4:00 P.M., September 15, 1934, beneath a piece of rotting log mold in a sugar-maple forest near Joliet, Illinois. The larva was large, measuring 18.6 mm. in length. It was moderately flattened and silphoid in shape and brilliantly colored. The antennae, mouth-parts, and legs were bluish-black; head and urogomphi and anal tube were orange; the thorax and abdomen were bluish-purple dorsally.

This larva was reared at laboratory temperature, in moist leaf mold. It molted on September 18, and a cream-colored pupa emerged from the cast larval exuviae. The pupa remained more or less active. It could vibrate the abdomen, and the abdominal apex could be elevated some 3 mm. if the rearing jar was struck sharply. The pupal period endured 192 hours, or 8 days, the male imago emerging about 6:00 P.M., September 23. This pupal period is some 2 days shorter than that reported by Balduf and probably represents more nearly the minimal duration. Other north-temperate records for carabid pupation agree in general; for example, Oertel (1924) found the European *Carabus granulatus* to pupate in from 7 to 9 days, and Classen (1919) reported that the American *Chlaenius impunctifrons* pupated in 7 days.

When the Joliet imago emerged, it was active and photonegative, dull in color, with the elytra cream tinted and the rest of the body and appendages a darker brown. By 8:00 A.M. of September 27, the adult had the typically purplish integuments of the species, and sclerotization was completed.

These simple data are of interest not only in themselves but as materials for a proper over-all explanation of the problem of distribution raised earlier. Why does *D. purpuratus* increase in average adult size from north to south?

Over its entire range the species inhabits rich deciduous woods. These forests vary in floristic and faunistic composition but agree generally in ecological position, namely, in their seral maturity. As we have seen, the adult is nocturnal, in the northwestern portion of its range at least, and both larval and adult stages are probably carnivorous. They must obtain their food from among the small ani-

mals of the forest floor, and time of feeding, duration of the feeding period, and foods eaten certainly enter into the problem.

In this discussion we differentiate between population density and the size of adult beetles from north to south. Adult population density may increase, decrease, or remain relatively constant from northern to southern portions of the range, but we have no data on this point. It is with adult size that we are concerned, although under certain conditions it is quite probable that larval population density might affect adult size.

Food would not seem to be the complete explanation for increase in size southward in the *purpuratus* population. It is difficult to see how increase in the number of kinds of foods southward would affect beetle size, unless in the southern areas there were particular food-animals that had a special biochemical effect when eaten by the larvae, e.g., a growth-promoting effect. Amounts of food for larval *purpuratus* in general would not tend to increase larval size because of the operation of temperature factors, to be noted presently. As a special case, amount of food might operate in exceptional circumstances to influence adult size. For example, if a larva were so situated that its food consumption was considerably below normal requirements, the resulting pupa, and the imago, might be unusually small. This would not be a general effect but might well explain the uncommon appearance of nonheritable dwarfism, noted previously.

Another factor involved is the activity pattern. If we assume that *purpuratus* larvae and adults are nocturnal over the range of the species (viz., not exhibiting the shift of activity pattern with latitude discussed by Krumbiegel for *Carabus nemoralis* in Europe), nocturnality would not serve to explain the observed shift in average size. The activity pattern of *purpuratus* is composite, containing both exogenous and habitual components. Since the length of the diurnal period (hours of sunlight) increases northward during the growing season, the nocturnal adults and presumably nocturnal larvae would have less and less time to find food from south to north in correlation with the shorter and shorter nights. This would not be a large change. It would amount to about 1½ hours less sunlight in June at the southern periphery of the range in contrast to the northern periphery. If this shorter nocturnal period to the north were effective, it would operate to produce smaller northern larvae, food-supply and other factors remaining optimal, and, actually, larger adults are found southward. Adjustment of feeding activity to the onset and duration of night is probably a factor.

Another factor to be considered is temperature. Mean air and soil temperature and length of growing season increase southward, other conditions remaining normal. Were we dealing with a poikilotherm that continued to grow as an adult, until the maximum size for the species was reached prior to natural death, a combination of food, activity-pattern adjustment, and temperature might explain the presence of larger adults from north to south. We are dealing with an insect, and these do not molt and grow when adult. Furthermore, in holometabolic forms such as beetles, the larger the last larval instar, the larger the pupa and, generally speaking, the larger the imago.

Now if the adult *purpuratus* were adapted in size to a given latitude, yet did not grow after reaching adulthood, such size variation would be genetic. This is not our position. We believe that the correlation of size with latitude is the

result of direct induction by the environment and is not hereditary. Consequently, as noted previously, it is with the pre-imaginal life that we should be concerned primarily in the solution of the problem as it affects adults.

Of cardinal importance here is the fact that, in general, the rate of developmental processes is accelerated with increase in temperature. This physiological factor would intervene. That is, even assuming that there were more kinds of foods and larger amounts of food available, the southern larvae would have less time in which to eat, from leaving the egg to forming the pupa. This should produce larvae that were either no larger, or even smaller, from north to south. The problem, then, begins to take on the aspect of what might be called "zoögeographic embryology."

Pertinent to this phase of the problem are data accumulated by Burgess (1911) on the carabid, *Calosoma sycophanta*. The incubation period for eggs varied from 3 to 10 days, the length of the period depending primarily upon incubation temperature. For example, in May the eggs hatched in an average of 7 days, in June 5.2 days, in July 4.4 days, and in August 4 days. Similarly, larvae that hatched in May, from eggs that were oviposited by females taken from hibernation in March and April, required an average of 5.5 days for the first instar, 6.5 days for the second, and 14.5 for the third. In contrast, larvae that hatched from eggs in June required 2 days for the first instar, 3 days for the second, and 9 days for the third. Similar examples are reviewed by Balduf (1935), and the application of such cases to the zoögeographic problem under examination is obvious but renders the explanation more difficult.

It would appear, then, that temperature is a primary factor, but with several effects. We must seek a factor complex that would produce a greater growth southward before size fixation, which occurs at the attainment of the imaginal state. With respect to temperature, this could be produced by altering the ratio x/y, where x is the effect of temperature upon the rate of larval-size increase and y is the temperature effect upon the duration of the pre-imaginal stages. Such a ratio should be subject to an experimental resolution, and other important factors that may be involved, such as light or special growth-promoting food factors, would probably operate in the same manner.

It is probable that the postulated increase in larval and pupal size from north to south involves both increased feeding time per night and warmer nights in which to feed during the growing season but that the thermal characteristics of physiological processes of metabolism, summarized by Heilbrunn (1943), also change with environmental temperature to allow more effective feeding periods. There is also the possibility that the influence of the growth and differentiation hormone and the pupation hormone are indirectly affected by external temperatures and play a part in the regulation of pupal size.

Over and above all these internal and external factors is the community factor. Since *purpuratus* is an inhabitant of rich deciduous forests, the blanketing effect of the stratified vegetation is very pronounced. During the growing season, the amount of change in light, air temperature, relative humidity, rate of evaporation, and wind movement would be relatively little from day to night, and relatively and actually small in the dark, damp log mold, where the beetles live. This obvious point is made because the over-all shift in the average size of adults, although gradual, is conspicuous when

viewed as a whole (Fig. 2); and if the physiological processes of development are affected by external conditions, such as a gradual change in north-south floor-mold temperatures, then they must be quite sensitive.

As with so many problems in biogeography, no complete explanation is available for the problem set forth here. Needed investigations include (1) a larger sample, especially material from 31° to 34° north latitude; (2) field work to check the altitude factor with reference to average adult size at a given latitude; (3) rearing of larvae through the pupation period with several conditions of temperature and food supply; (4) experimental analysis of the activity pattern from several well-separated regions of the range.

SUMMARY

The average adult size of individuals of the population of the carabid beetle, *D. purpuratus* Bonelli, increases from northern to southern portions of its range in eastern North America, through about 14° of north latitude.

This exemplifies that part of the Bergmann zoögeographic principle that has to do with poikilothermal animals.

Since adult size is fixed at the end of the pupal stage, the burden of any explanation rests on the pre-imaginal period.

Community-type inhabited, habitat niches occupied, life-history events, qualitative and quantitative aspects of food supply, adjustment of nocturnal activity pattern to change in ratio of day to night, environmental temperatures, thermal characteristics of developmental processes, and hormones of the developmental period may be involved in the explanation. The explanation is complicated by the fact that generally the rate of developmental processes is found to increase with rise in temperature within certain limits.

One solution offered, capable of future experimental resolution, is that of an altering of the ratio x/y, where x is the effect of temperature upon the rate of larval-size increase and y is the temperature effect upon the duration of the pre-imaginal stages.

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INTEROCULAR TRANSFER OF VISUAL DISCRIMINATION HABITS IN A TELEOST FISH[1]

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IN CONDUCTING experiments on the retention of visual discrimination habits following optic nerve transection and regeneration in teleosts, it became desirable to know whether habits learned with one eye would transfer without additional training to the other eye. It has been shown that a habit established with one portion of either retina in man is retained when any other part of the two retinas is used (Becher, 1911). In unpublished studies (see Levine, 1945*a*) contralateral transfer has been found in the rat for brightness and pattern discriminations and in the chicken for discrimination of two shades of gray. On the other hand, Beritov and Chichinadze (1937) found a lack of transfer of color discrimination in the pigeon. This was confirmed by Levine (1945*a*, *b*) for color and pattern discriminations involving the lower temporal part of the retina. Transfer was obtained, however, from the upper temporal part of the retina. Levine observed further that pigeons may learn a visual discrimination with only one eye, even though neither eye is blindfolded. The results on the pigeon show that interocular transfer cannot be assumed a priori for the lower vertebrates and that further information is needed.

What the presence or absence of interocular transfer may mean with respect to the neural basis of sensory equivalence, learning, and memory has yet to be determined. It was thought that the goby, a marine teleost, might be a good test animal for further analysis of the problem. The goby learns visual discrimination habits without excessive training; it can be effectively blinded for long periods with opaque eye caps; its optic nerves can be severed or its eyes transplanted with recovery of vision (Sperry, 1948, 1949); and its brain is simpler and amenable to more drastic surgery than that of warm-blooded vertebrates. Also this ani-

[1] The experiments were conducted at the Lerner Marine Laboratory of the American Museum of Natural History, at Bimini, British West Indies. They were aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

mal is hardy, easily secured and maintained, and, from the viewpoint of training, its relative immunity to affective disturbances from noise and movement in its surroundings and its tendency to remain on the bottom between trials constitute important advantages. As in other infra-mammalian vertebrates, the crossing of fibers in the optic chiasma is complete.

In the following experiments a group of these fish were taught a simple visual discrimination with a blinder on one eye. After the fish had been overtrained, the blinder was shifted to the opposite eye and the degree of transfer determined. In cases in which transfer was poor or lacking, the blinders were shifted once or twice more and the test performances for each eye compared. In a second series of fish, monocular performances were tested in a habit which had been acquired with both eyes uncovered.

MATERIALS AND METHODS

One of the gobies, *Bathygobius soporator* (Cuvier and Valenciennes) was used throughout. The specimens measured 24–43 mm. in standard length and were collected during February from tide pools on North Bimini. After a blinder had been placed on the right eye as described below, each fish was placed in a 2-gallon aquarium, 16 × 20 × 26 cm., with standing sea water that was changed approximately every 4 days. Two days were allowed for the fish to become hungry and accustomed to their aquaria before training was started.

These fish, instead of swimming about freely, spend most of their time clinging to the bottom and sides of the aquarium by means of their specialized pelvic fins. The training consisted of teaching the fish to swim to the higher of two stimulus objects presented simultaneously from above. The positive stimulus, a piece of conch, fish, or crab meat, approximately 5 mm. in diameter, was hung on stainless-steel wire, 35 mm. directly above a knotted wad of glass wool, or in other cases a piece of red rubber, about twice the size of the positive stimulus. There was an initial preference for the lower, larger object. At first the lures were set deep near the bottom and were left for several minutes until the fish overcame their timidity and explored the double lure. As training progressed the stimuli were presented at successively higher levels and were removed more quickly until, by the fifth day of training, merely dipping the lures into the water was sufficient to elicit an immediate response. Thereafter, the presentation was made randomly at various depths but, in the majority of trials, above the starting level of the fish.

If, in approaching the lures, the fish committed themselves in any way to an initial selection of the negative rather than the positive stimulus, even though an actual bite was not made, it was counted an error. The only punishment for an error was an abrupt withdrawal of the lures. When a correct trial was made, the lures were withdrawn gently after allowing the fish to nibble once or twice at the bait. An average of 30 trials was given per day after the fish had started to run well, 15 in the morning and 15 in the afternoon, with the individual trials in each series separated by intervals which varied from approximately 1/2 to 3 minutes.

Monocular blinding was accomplished by fitting an opaque cap of tantalum foil over one eye. The foil was first molded into a cup shape having approximately the curvature of the eye and then cut to fit the size of the eyeball. This cap was inserted between the eyeball and the free external cornea through an incision in the skin dorsal to the cornea just large

enough to permit passage of the cap. The diameter of these blinders was slightly less than that of the eyeball but greater than the longest dimension of the pupil, so that the latter was completely covered. The blinders did not impair eye movements and caused little or no mechanical inconvenience. In the majority of cases the incisions healed rapidly, sealing the blinders in place without infection or clouding of the cornea. In shifting the blinders from one eye to the other, the original incision was reopened, and the cap was pulled out and placed over the opposite eye in the same manner. These operations were performed with urethane anesthesia and always at night, in order that any affective disturbance might be dissociated as much as possible from the daytime training procedure.

Various measures were taken to rule out possible use of unintended nonvisual cues. Any dependence upon constant spatial cues was eliminated by presenting the bait randomly at different levels and in different parts of the aquarium. Use of olfactory cues was checked during the overtraining sessions by using a stale, washed-out piece of conch for bait and excessively flavoring the aquarium water with fresh conch tissue fluids. All the fish continued to run correctly under these conditions with no signs of confusion. Also when a wad of cotton of comparable size was substituted for the bait following the test trials, the fish continued to run directly to the cotton for as many as 7–12 consecutive trials. Upon presentation of the lures outside the glass walls of the aquarium on scattered trials during the overtraining sessions, the fish responded in the usual way and attempted to get the bait through the glass. With the lures outside the glass, it was not always possible to determine satisfactorily which one of the two stimulus objects was being aimed at, however, unless the approach was approximately horizontal. Only 3–4 trials of this latter sort were obtained per individual, but the manner in which the fish responded, as well as the bare scores in these tests through the glass, indicated that the habit was not dependent upon either chemoreceptor or lateral-line cues. The fact that the fish frequently approached the bait from above as well as from below in the later stages of training also tended to rule out lateral-line guidance.

Many fish with one eye blinded will easily get into the habit of approaching the bait with a partial turn toward the seeing side, a motor pattern which, in itself, might hinder performance with the other eye. Such turning was decreased by frequently placing the lures on a straight line in front of the fish. Especially in the final stages of overtraining, care was taken to be sure that the animals ran correctly with the bait directly in front. In the test trials the bait was always presented straight in front as close as possible to the mid-sagittal plane.

Near the end of the overtraining sessions each fish was netted, anesthetized with urethane, and manipulated as would be done in the course of an operation except that no surgery was performed. This was found to have no significant influence upon the training scores or upon the animals' readiness to run on the following morning.

RESULTS

Training was started with a group of 22 animals, 4 of which had to be discarded because they refused to run and a fifth because it failed in 12 days to reach a criterion of 17 correct out of 20 consecutive trials. The remainder attained this criterion in 3–7 days of training. The first 6 cases listed in Table 1 were trained with glass wool as the negative stimulus. In these the learning time averaged at least

2 days longer than in the others for which red rubber was used as the negative stimulus. The white color and general contour of the glass wool was very close to that of the bait, whereas, in the case of the rubber, color and form contrasts were present, in addition to the position and size differences.

After the learning criterion had been reached, the animals were overtrained for about 4 days or a minimum of 120 trials. The blinder was then shifted from the right to the left eye. During the 2 days preceding this shift of the blinder, the fish were running on the average about 85 per cent correct. The individual error scores for the final 25 trials immediately preceding the transfer of the blinder are shown in Table 1.

Although some of the fish responded readily on the morning following the shift of the blinder, others did not start to run again until the second or third day. There was no significant correlation, however, between this hesitancy to run and the degree of transfer of visual discrimination. One animal had to be discarded because its eye became infected and it refused to feed. In the remaining 16, a total of 25 trials was obtained with the left eye covered. The number of errors made by each is shown in the table. Apparently, transfer was almost complete in some animals (5, 9, and 12), but quite poor or negligible in others (2, 4, 7, 8, 11, 13, and 15).

In those fish in which transfer was poor or lacking, the blinder was shifted back again to the right eye. Case 11 became infected and refused to run. In the remaining 10 cases, 25 trials were obtained, and the scores all showed an abrupt, marked improvement to approximately the level attained previously with the right eye covered. It is possible that slowly developing infections, which became worse after the 25 trials had been obtained, served to increase the number of errors in cases 2 and 4. A third shift of the blinders back to the left eye was undertaken in Nos. 3 and 7; No. 3 again showed only poor transfer, whereas No. 7, in contrast to its earlier performance, this time exhibited good transfer.

In a second series of fishes trained for other experimental purposes, some incidental observations were obtained pertaining to the question of whether learning may be uniocular, as in the pigeon, even though neither eye is blinded during training. Fifteen animals were trained in the same way as above, except that both eyes were left uncovered. In all cases the negative stimulus was glass wool. Two cases did not run readily and were discarded. After the remaining 13 had been overtrained in 60–90 trials, a tantalum blinder was placed on the right eye, and 25 trials were given. In 6 of these cases the blinder was then switched to the left eye and 25 trials given with this eye covered. Finally, in these 6 and in 5 others the blinders were removed and 25

TABLE 1

NUMBER OF ERRORS MADE IN THE 25 TRIALS PRECEDING AND FOLLOWING TRANSFERS OF THE BLINDER IN SERIES I

Case No.	Eye Blinded			
	Right	Left	Right	Left
1	3	12	4	.....
2	5	20	11	.....
3	2	13	3	10
4	6	21	7	.....
5	5	6	.....	.....
6	4	8	.....	.....
7	3	19	5	5
8	2	18	2	.....
9	1	2	.....	.....
10	0	11	3	.....
11	2	22	.....	.....
12	5	6	.....	.....
13	3	18	4	.....
14	3	7	.....	.....
15	2	20	1	.....
16	2	10	4	.....

trials were given with both eyes uncovered. The use of the fish in this series for another experiment prevented further testing for monocular dominance. The error scores obtained are shown in Table 2.

The level of monocular discrimination was clearly too high in the group as a whole to support the supposition that learning and retention had been confined to one eye. Furthermore, high error scores in the last column of the table indicate that affective disturbance became an appreciable factor in this series in which the amount of overtraining had been less than in Series I. Accordingly, the impairment which can be attributed to unilateral blinding alone is probably somewhat less than indicated by the scores in the table.

TABLE 2

NUMBER OF ERRORS MADE IN 25 TRIALS PRECEDING AND FOLLOWING INSERTION AND REMOVAL OF THE BLINDERS IN SERIES II

Case No.	Eye Blinded			
	None	Right	Left	None
1	6	9	.....	4
2	0	15	11	1
3	3	8	9	7
4	3	16	.....	2
5	4	13	6	3
6	3	3	.....	.....
7	4	7	10	15
8	4	12	4	8
9	2	1	12	6
10	1	4	.....	.....
11	2	3	.....	2
12	3	3	.....	6
13	4	9	.....	2

DISCUSSION AND CONCLUSIONS

From the sudden drop in level of performance produced in the majority of cases by shifting the blinder from the untrained to the trained eye in Series I *plus* the consistent return to a high level of discrimination when the blinder was shifted back again to the untrained eye, it may be inferred that interocular transfer does not take place in a complete and automatic fashion in these teleosts as it does in man. The untrained eye, in the majority of cases, is not always equally as good as the trained eye for a learned visual discrimination.

That the data might be accounted for alternatively in terms of generalized affective or motor disturbances associated with shifting the blinders seems implausible in view of the following: Affective disturbance would appear to be ruled out by the consistent improvement produced by the second shift of the blinders to the untrained eye and also by the apparent intrepidity with which many of the animals performed. Dependence upon a lateral bias in motor patterns was excluded by means already described. That the presence of the blinders over the eyes caused any regressive or atrophic changes in the visual system that might have been responsible for lack of transfer seems improbable. Some animals showed good transfer on the day after the blinders were shifted, while others that did not respond until the second and third days failed to show transfer.

On the other hand, from the high degree of transfer that did take place in a minority of cases it may also be inferred that interocular transfer is by no means impossible in these fish. The question arises in this regard as to whether the apparent interocular transfer in these instances might not have been based upon nonvisual cues. Certainly, many aspects of the habit were not visual and accordingly would be expected to survive the shift of the blinders. However, that component of the habit which involved the initial localization of the lure in space is something for which vision was indispensable. Gobies which have been trained to rise to such a lure are no longer

able to locate it after bilateral blinding, even though, with olfactory excitation, they may dart about in obvious search for it. This conditioned response to swim directly to the lure upon its presentation, exclusive of any discrimination between its positive and negative portions, transferred in varying degree in all cases, even in those animals in which the discriminatory component failed to transfer.

That transfer of the discrimination itself might have been dependent upon olfactory and lateral-line rather than upon visual cues seems to be discounted by various considerations already mentioned to which the following may be added: The direct course taken in the approach to the lure and the speed of the proach when the animals were running correctly indicated that, in general, the discrimination had already been made before the fish got close enough to use any olfactory or lateral-line cues. Slow, hesitant approaches, in which the fish did not commit themselves until they were close to the lure, were correlated with poor scores rather than with good scores. It was not uncommon for a fish which was running poorly to approach to within 2–3 cm. of the lure and, after hovering in this position for some time, to strike finally at the glass wool or rubber. Although absolute proof is lacking that a combination of lateral-line and chemical cues may have been responsible for the transfer, the evidence, on the whole, is against such an interpretation.

In the course of the training period the majority of the blinders slipped a little from their original position. In some cases this was sufficient to allow a thin crescent of peripheral vision, usually downward. However, there was no correlation with this accidental peripheral vision in the covered eye and the extent of transfer. In none of those in which transfer occurred was the peripheral vision upward or forward. In two of them there was no peripheral vision at all in the covered eye. Among the group that did not transfer, on the other hand, were two cases with slight peripheral vision upward and forward. It should be recalled that the blinders moved with the eyeball, and therefore a thin opening under the edge of the blinder could not be utilized to the extent that is possible with external blindfolds of the type used on birds and mammals.

In summary, the results in Series I point to the conclusion that monocularly acquired visual discrimination habits are subject to contralateral transfer in the goby, i.e., the organization of this teleost brain does not preclude interocular transfer. At the same time, however, it must also be concluded that the neural mechanisms are not so well developed that they consistently insure complete and automatic transfer in all instances, as in man.

It was not possible to correlate the lack of transfer with any particular factor, such as apparent timidity or slowness in approaching the lures, extent of overtraining (within the limits of the experiments), slowness of learning, etc. Nor was there anything to suggest that those fish which showed interocular transfer had learned the discrimination with a different part of the retinal field from that used by those in which transfer failed to occur. Further evidence on this and other points would be desirable before trying to assess the implications of the data for problems concerning the neural basis of visual learning, memory, and sensory equivalence.

In Series II the degree of impairment of the binocularly acquired habit caused by the unilateral blinding was much too small for the group as a whole to be compatible with the assumption that the discrimination had been learned exclusively

by one dominant eye with no interocular transfer. If learning and retention had been confined to one eye, approximately half the fish should have shown severe or complete loss of correct discrimination when the right eye was blinded, and the remainder ought to have shown similar loss when the other eye was blinded. Possible influences upon the error scores caused by affective or other generalized disturbances associated with insertion of the blinders would serve only to strengthen the foregoing inference.

SUMMARY

1. Inserted blinders of tantalum foil were devised for monocular training of visual discrimination habits and the testing of interocular transfer in fishes.

2. Sixteen gobies with a blinder on the right eye were trained against an initial preference to swim to the smaller, higher, and less brightly colored of two lures presented simultaneously. Controls were run to rule out the use of nonvisual cues.

3. After the fish had been overtrained 4 days or a minimum of 120 trials, the blinder was shifted to the opposite (left) eye. Retention tests revealed excellent or good interocular transfer in 5 of the 16 cases. Lesser degrees of transfer were evident in 4 cases, while in the remaining 7 the transfer was poor or negligible. When the blinder in these last 11 cases was shifted again to the untrained (right) eye, the scores promptly underwent a marked improvement to approximately their previous level.

4. An additional shift of the blinder to the trained (left) eye in 2 cases again caused deterioration of the habit in 1 of them. In the other, however, in contrast to the earlier result in the same individual, excellent transfer was manifest.

5. In a second series of 13 fish, monocular performances on visual discrimination habits learned with both eyes uncovered indicated that learning and retention had not been confined to one dominant eye as found in some earlier experiments with pigeons.

6. The results seem to support the conclusion that the brain organization of this teleost fish permits interocular transfer, but that, at the same time, the neural mechanisms involved are not so well developed that good transfer is automatically assured in all instances.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

BIOCHEMICAL EVOLUTION[1] BY MARCEL FLORKIN; EDITED, TRANSLATED, AND AUGMENTED BY SERGIUS MORGULIS

Reviewed by AURIN M. CHASE
Princeton University

As THE translator states in his preface, this book is not an exact rendition of the original French version, some material having been omitted and some added at his discretion, with the approval of the author. Twenty-eight of the 157 pages are given over to a section containing a list of references, a short zoölogical classification, author and subject indexes, and an index of animals referred to in the text. This section seems relatively large for a volume of this size and should contribute appreciably to the usefulness of the book. The 300-odd references cited include about equal numbers of French, German, and English titles, with a few in other languages. About four-fifths of them are prior to 1940. The factual material discussed is taken almost entirely from experimental work on animals, so the book may be of more value to zoölogists than to botanists.

The first two chapters present a consideration of the apparent unity of the biochemical plan of animals but also of some of the known dissimilarities. Chapters iii and iv are devoted to a comparison of such functions as oxygen transport, phosphate and electron transfer, enzymatic processes of digestion, and protein metabolism throughout the animal kingdom. The evolution of biochemical constituents is discussed in connection with some of these functions.

Chapter v is divided into a number of sections, each dealing with a particular "domain," e.g., the domain of respiratory function, those of digestion, photoreception, etc. A great deal of detailed information is presented in this chapter concerning specific organisms and their adaptations for life under particular ecological conditions. Indeed, so much material is given that it is sometimes difficult to follow the main line of thought.

Chapter vi, "Systematic Characters," presents a summary of biochemical characteristics of different groups of animals. A classification of animals is suggested on the basis of such characteristics as amino acid content of the blood or body fluid, type of respiratory pigment, type of phosphagen present, etc. One gets the impression (not intended by the author) that, while such a classification may ultimately be possible, the biochemical data presented are not sufficiently uniform or reliable for such a purpose except in certain isolated cases. This does not imply, however, that such a classification may not eventually be justified.

The last chapter, "Perspectives," in the words of the author (from the Foreword of the book), ". . . sums up some inferences which are still largely hypothetical but which give a clue to the task yet to be accomplished and which each can judge according to his own tastes." This chapter specifically presents the arguments for the thesis that, in the last analysis, evolution is directed by biochemical phenomena. It seems hardly likely that many physiologists or biochemists would disagree with this proposition, even though the evidence for it is incomplete at the present time. Whether or not the author's arguments are accepted, the book contains a very useful collection of data on the comparative biochemistry of animals which should be of value.

In the opinion of the reviewer, the text as a whole suffers from a certain amount of repetition, and sometimes comprehension is made difficult by the use of unnecessarily involved

[1] New York: Academic Press, Inc., 1949. Pp. vii + 157. $4.00.

phrases and sentences. There is also an occasional tendency to employ terms which are seldom encountered in current biochemical literature. These features can quite possibly be attributed to difficulties of translation, but they do not improve the readability of the book.

It is difficult to avoid comparing this book with that by Baldwin, *An Introduction to Comparative Biochemistry*, which covers much the same field, though less exhaustively and with less direct emphasis on the evolutionary theme. If the present volume had, in addition to its reference value, the pleasant style of presentation of Baldwin's, it would be improved.

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